Regulation of Sphingosine Kinase 1 Signalling by Calcium- and Integrin-binding Proteins

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Chapter 1: Introduction

Sphingosine kinase 1 (SK1) is a lipid kinase that phosphorylates sphingosine to form sphingosine -1-phosphate (S1P). This reaction lies within the sphingomyelin cycle in cells, where the basal activity SK1 is proposed to balance cellular levels of its upstream pro-apoptotic mediators, ceramide and sphingosine, with the pro-survival and pro-proliferative signalling of its product S1P (Spiegel and Milstien, 2003b). However, SK1 also has an active role in cellular signalling pathways, with this enzyme transiently activated by a number of agonists at the cell surface, generating a greater supply of S1P. As high levels of S1P promote cell proliferation, protection from apoptosis and neoplastic transformation, there has been much resent focus on the role of SK1 in cancer, and manipulation of this enzyme for cancer therapeutics.

The agonist-induced transient activation of SK1 is regulated through phosphorylation of the enzyme at Ser225 by ERK1/2 (Pitson et al., 2003; Pitson et al., 2005), with phospho-SK1 then translocating from the cytosol to the plasma membrane. Both the phosphorylation and translocation of this enzyme are critical in SK1-induced tumourigenesis (Pitson et al., 2005). This thesis examines the regulation of SK1, and in particular the control of SK1 translocation to the plasma membrane.

1.1 Sphingosine-1-Phosphate as a Signalling Molecule

Sphingolipids are simple molecules, composed of a fatty acid linked to a sphingoid long-base chain through an amide bond. These molecules are structural components of all eukaryotic membranes. At the plasma membrane, these lipids are believed to form a stable and chemically resistant outer leaflet of the lipid bilayer, and have a protective effect on the cell surface (reviewed in Takabe et al., 2008). However, sphingolipids have also been shown to often act as bioactive signalling molecules, influencing a wide array of signalling events. One of the most widely studied sphingolipids is sphingosine-1-phosphate (S1P), with this phospholipid influencing a diverse array of cellular processes, including proliferation (Zhang et al., 1991; Olivera and Spiegel, 1993), migration (Wang et al., 1999;

Lee et al., 2001; Graeler et al., 2002), survival (Cuvillier et al., 1996), angiogenesis (Lee et al., 1999; Liu et al., 2000b) and immune cell trafficking (Lin and Boyce, 2006; Olivera and Rivera, 2005). It is thought that S1P can function in such diverse signalling roles through actions both within and outside of the cell (reviewed in Takabe et al., 2008). Although the intracellular targets for S1P are only beginning to be revealed, extracellular S1P has been well established to act as a ligand for five specific G-protein coupled receptors, termed $S1P_{1-5}$ (reviewed in Pebay et al., 2007). Thus, the coupling of each of these receptors to various G proteins and their numerous downstream signalling pathways is thought to regulate many of the diverse physiological processes governed by S1P in a highly cell-specific manner (reviewed in Taha et al., 2004).

In addition to the above mentioned cellular processes, an overproduction of S1P has been linked to various pathophysiological conditions (reviewed below). The most widely studied of these is cancer (Ogretmen and Hannun, 2004; Padron, 2006; Sabbadini, 2006). Hence, it is thought that S1P may be a potential target for treatment of these diseases, with much research focus now on the regulation of S1P generation and its downstream signalling events.

1.2 The Sphingolipid Metabolic Pathway

Sphingosine-1-phosphate is generated by the activity of sphingosine kinase (SK), with this reaction part of the 'sphingomyelin cycle' in cells (Figure 1.1). Degradation of sphingomyelin to ceramide, followed by deacylation of ceramide to form sphingosine, provides the substrate for this conversion. S1P lyase then can degrade S1P to the smaller molecules hexadecenal and ethanolmine phosphate, in essentially the only irreversible reaction in this pathway. Alternatively, S1P can be dephosphorylated, converting it back to sphingosine, by either of two specific S1P phosphatases, SPP1 and SPP2 (Ogawa et al., 2003; Le et al., 2002). S1P can also be non-specifically dephosphorylated by a family of at least three broad specificity lipid phosphate phosphatases, LLP1, LPP2 and LPP3, with these enzymes also capable of dephosphorylating phosphatidic acid and ceramide-1-phosphate (reviewed in Pebay et al., 2007). Sphingosine can only be directly produced through the recycling of these sphingolipids. Ceramide, on the other hand, can also be produced by *de novo* synthesis.

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This figure is included on page 2a of the print copy of the thesis held in the University of Adelaide Library.

(Adapted from Pitman and Pitson, 2010)

Figure 1.1. The Sphingomyelin Cycle regulates intracellular levels of S1P. Sphingomyelin is a constituent of lipid rafts in the plasma membrane. This can be degraded to ceramide and then sphingosine providing the substrate for Sphingosine Kinase. SK can then convert this to S1P. Degredation of S1P to the smaller molecules hexadecenal and phosphoethanolmine is mediated through S1P lyase, in essentially the only irreversible reaction in this pathway.

The *de novo* synthesis of ceramide can be induced by a number of different factors, including hypoxia, tumour necrosis factor alpha (TNF-α), and some chemotherapeutic agents (reviewed in Takabe et al., 2008). This *de novo* biosynthesis pathway occurs mainly at the cytosolic-leaflet of the endoplasmic reticulum (ER), where the enzymes required for ceramide synthesis are located (Takabe et al., 2008). The initial reaction involves the condensation of L-serine with palmitoyl-CoA, catalysed by serine palmitoyltransferase, to form 3-ketosphinganine (Merrill, Jr., 2002). This is the rate limiting step of the biosynthetic pathway (Merrill, 2002). 3-ketosphinganine can then be reduced to dihydrosphingosine followed by N-acetylation to form dihydroceramides. These are then converted to various chain-length ceramides by a desaturase. For sphingomyelin synthesis, ceramide or dyhydroceramide are transported from the ER to the Golgi. This is mediated through nonvesicular transport by CERT, a ceramide transport protein (Hanada et al., 2003). Ceramides or dihydroceramides are converted to sphingomyelins by sphingomyelin synthase on the luminal side of the golgi or to glucosylceramides (GlcCer) on the cytosolic surface of the Golgi respectively (van and Holthuis, 2000).

Interestingly, the two specific S1P phosphatases, SPP1 and SPP2 are localised to the ER, with their catalytic sites predicted to face the luminal side of this organelle (Ogawa et al., 2003; Le et al., 2002). The sphingosine formed at this location by these phosphatases appears to become the substrate for ceramide synthesis, by the ceramide synthases present on the cytosolic face of this organelle (Le et al., 2002). Hence, these phosphatases are proposed to assist in regulating the balance between S1P and ceramide levels (Le et al., 2002).

This sphingolipid metabolic pathway has been recognized as much more than a degradative pathway for sphingomyelin, since many of the intermediates have been identified as signalling molecules, often with quite contrasting effects on cells. While elevated levels of S1P enhance cell survival and growth in diverse cell types, its precursors, sphingosine and ceramide have generally been associated with cell growth arrest and apoptosis (Maceyka et al., 2002; Woodcock, 2006). As these metabolites are interconvertible, it has been suggested that it is their relative levels rather than absolute amounts that regulate cell fate. Consequently, SK lies at a critical junction in this pathway, as its activation not only produces higher levels of S1P, but concurrently reduces levels of sphingosine and ceramide. Hence, this has been referred to as the sphingolipid rheostat (Spiegel and Milstien, 2003b).

1.3 Enzymology of SKs

Sphingosine kinases have been cloned from a number of different organisms including *Sacharomyces cerevisiae* (Nagiec et al., 1998), *Arabidopsis thalana* (Imai and Nishiura, 2005), *Drosophila* (Herr et al., 2004), rat (Imamura et al., 2001), mouse (Kohama et al., 1998) and human (Pitson et al., 2000a; Melendez et al., 2000; Nava et al., 2000; Liu et al., 2000a). These enzymes are evolutionarily well conserved, sharing similar biochemical properties and displaying comparable kinetic parameters. Together with the ceramide kinases, the SKs posses five conserved regions in their polypeptide sequence designated C1-C5 that are unique to these enzymes and can be used to define this family of proteins (Figure 1.2) (Baumruker et al., 2005; Leclercq and Pitson, 2006). Aside from sharing some similarity with the putative catalytic domain of the diacylglycerol kinases, acylglycerol kinase and ceramide kinases, the complete SK sequences are quite distinct from any other known proteins or protein domains, making predictions of regulatory mechanisms, possible binding partners and cellular localisation difficult. In addition, the SK sequences show no clear homology to the well established ATP binding motifs of other kinases; Gly^{82} and Lys^{103} have, however, been identified as essential residues for nucleotide binding in human SK1, and a motif of $SGDGx_{17\text{-}21}K/R$ has been suggested to represent the nucleotide binding region of the SKs (Pitson et al., 2002). This nucleotide binding region is quite unique to this family of proteins, sharing only weak amino acid sequence similarity to the highly conserved glycine-rich loop motifs of protein kinases and phosphatidylinositol phosphate kinases (reviewed in Wattenberg et al., 2006).

Using molecular cloning, two mammalian isoforms of SK have been identified, designated as SK1 and SK2. Despite these two enzymes originating from different genes (residing on chromosome 17 and 19, respectively) and differing quite substantially in size (43 and 65 kDa, respectively), they share a high degree of sequence similarity (Wattenberg et al., 2006). Almost all of the SK1 polypeptide sequence aligns with regions of the larger SK2, with an overall identity of 45% (80% similarity) (Liu et al., 2000a). SK2 however, possesses two additional polypeptide regions, one at its N-terminus and the second in the middle of its sequence, that are quite distinct from not only SK1, but also from any other protein (Figure 1.2). Within this central region of SK2 resides a proline-rich region predicted to bind SH3 domains, and four putative transmembrane domains, all lacking in SK1 (Spiegel and Milstien, 2002).

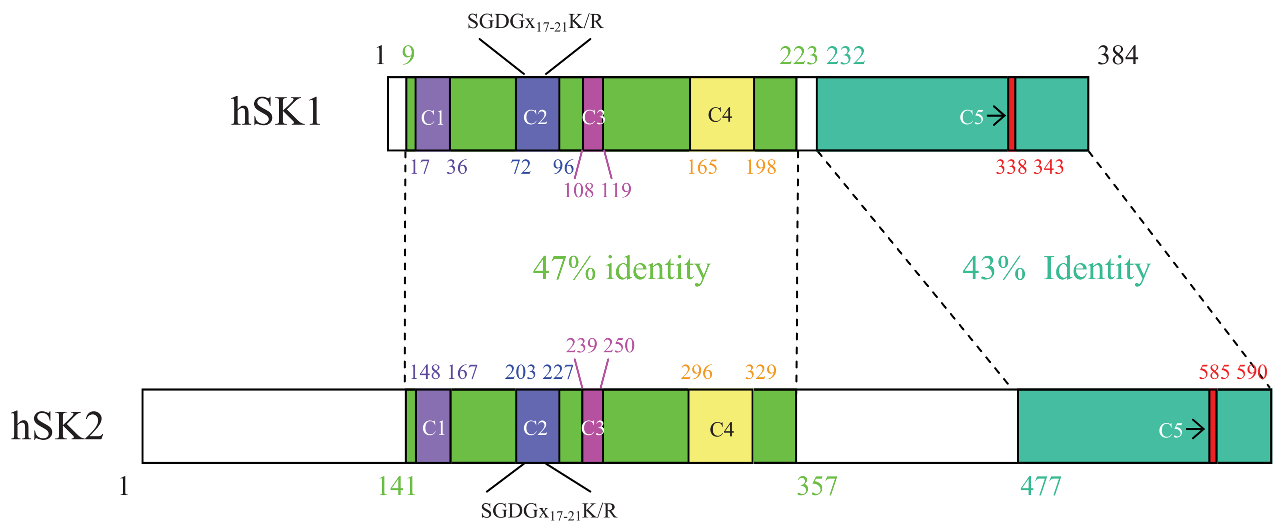

Figure 1.2. Two isoforms, SK1 and SK2 exist for human sphingosine kinase

A schematic representation of human SK1 and SK2, highlighting their conserved regions and the sequence identity in these regions. Mammalian SK1 and SK2 have five conserved regions (C1-C5). Also shown is the conserved ATP binding sequence SGDGx_{17-21}K/R.

In addition to these sequence disparities, SK2 has an approximate10-fold lower specific activity than SK1 (Roberts et al., 2004b), and appears somewhat more promiscuous than SK1 in the substrates it can utilize. Both SK1 and SK2 can efficiently use D-*erythro*-sphingosine and D-*erythro*-dihydrosphingosine. SK2, however, has a much greater activity towards phytosphingosine (Liu et al., 2000a; Pitson et al., 2000a) and an artificial substrate, ω-biotinyl D-*erythro*-sphingosine compared to SK1 (Roberts et al., 2004b). To further add to these disparities, L-*threo*-dihydrosphingosine, an inhibitor of SK1 (Kohama et al., 1998; Pitson et al., 2000a), is phosphorylated by SK2 (Liu et al., 2000a). Further studies have also indicated SK2 to be the main enzyme responsible for phosphorylation and activation of FTY720, with SK1 appearing to show little activity toward this immunosuppressive agent and related analogs (Billich et al., 2003; Paugh et al., 2003; Sanchez et al., 2003). The physiological significance of these differences in substrate specificity remains unknown.

In addition to their differential substrate preferences, SK1 and SK2 have different developmental expression and exhibit differential tissue distribution. While SK1 is most abundantly expressed in the lung, spleen and liver, SK2 is predominantly found in the liver and heart (Kohama et al., 1998; Liu et al., 2000a). These differences suggest that SK1 and SK2 may be differentially regulated to perform distinct functions. Furthermore, SK1 and SK2 appear to play quite contrasting roles in the cell. While SK1 promotes cellular survival and proliferation, overexpressed SK2 appears to have a pro-apoptotic effect on cells (Maceyka et al., 2005). Although the reasons for these opposing roles are still under examination, the distinct cellular localisations of the two isoforms may offer some clue to determining their function, with SK1 localized predominantly in the cytoplasm (Kohama et al., 1998; Olivera et al., 1999) and SK2 residing in the nucleus and at the ER (Igarashi et al., 2003; Maceyka et al., 2005). However, while these cellular studies favour differential roles for SK1 and SK2, this is still contentious, as both SK1 and SK2 single knock-out mice appear to display no obvious defects (Allende et al., 2004; Kharel et al., 2005), while SK1/SK2 double knock out mice die *in utero* (Mizugishi et al., 2005). This suggests compensation may occur with these enzymes.

1.4 S1P Signalling

The production of S1P has a very well defined pro-proliferative and anti-apoptotic effect in cells. As previously mentioned, cellular levels of S1P are largely controlled through its formation from sphingosine by the activity of SK, and to a lesser extent by its degradation by S1P lyase and S1P phosphatases. In a basal state, this balance between S1P generation and degradation generally results in low cellular levels of S1P (Spiegel et al., 1998). However, the activity of SK1 can be rapidly increased by exposure of the cell to a number of different agonists (described below), resulting in increased levels of S1P. This elevated level of cellular S1P has been shown to protect cells from apoptosis induced by various conditions including serum-deprivation (Olivera et al., 1999), ceramide (Maceyka et al., 2002), and TNF-α (Xia et al., 1999b; Xia et al., 2002). Overexpression of SK1 has been shown to result in greater proliferation coupled with decreased apoptosis in cells (Pitson et al., 2005; Xia et al., 2000). As the effect of SK1 overexpression in these cells is mimicked by the addition of exogenous S1P (Maceyka et al., 2002; Olivera et al., 1999) and inactive SK1 does not give this effect (Xia et al., 2000), one can assume the pro-proliferative, anti-apoptotic effects of SK1 are mediated directly through its production of S1P.

As well as this role in cell growth and survival, elevated levels of S1P have been implicated in an incredibly diverse array of additional signalling events. One such event is calcium mobilisation, with increases in cytosolic Ca^{2+} concentration widely observed upon activation of SK1 (Spiegel and Milstien, 2003b). Other signalling events of S1P include cytoskeletal rearrangement, mitogenesis, inflammatory responses and oncogenesis. The basis for these diverse actions of S1P has been proposed to occur through potential dual actions of this phospholipid, acting both inside and outside of cells.

S1P is present in high levels in the circulating blood stream, with human serum and plasma levels predicted to be 0.4-1.2 μM and 0.2-0.5 μM respectively (Okajima, 2002). This S1P is thought to originate from several different sources, including its release from platelets upon activation (Yatomi et al., 2001). More recent evidence has also implicated red blood cells as a major source of S1P (Pappu et al., 2007), but a diverse range of other cells, including the vascular endothelium (Venkataraman et al., 2008), have also been shown to release S1P, generally in response to cell stimuli, such as growth factors and cytokines that activate SK1 (Pebay et al., 2007). The release of S1P from cells can be mediated by ATP binding

cassette (ABC) transporters, with ABCC1 specifically identified as an S1P transporter in mast cells (Mitra et al., 2006). An ABC transporter has also been identified as mediating S1P from the cytoplasm of rat platelets (Kobayashi et al., 2006), while the ABCA1 transporter is critical for the release of S1P from astrocytes (Sato et al., 2007). More recently, an S1P transporter in Zebrafish and *Drosophila* has been identified as Spinster 2 (*two of hearts*) (Osborne et al., 2008; Kawahara et al., 2009), a member of the Spinster-like family of putative membrane transporters.

The release of S1P from cells acts to signal in both an autocrine and paracrine fashion, with S1P becoming a ligand for a family of five G protein-coupled receptors on the cell surface termed $S1P_{1\text{-}5}$. These receptors appear to bind only S1P and dihydro-S1P with high affinity. Differential expression of the various S1P receptors and their coupling to different G proteins is believed to regulate numerous downstream signalling pathways, allowing extracellular S1P to regulate diverse physiological processes (Figure 1.3). $S1P_1$ is ubiquitously expressed and appears to couple only to G_i proteins. Engagement of this receptor can activate several pathways, including phospholipase C (PLC), Ras, followed by extracellular signal regulated kinase 1/ 2 (ERK1/2) to enhance cell proliferation, phosphatidylinositol-3-kinase (PI3K)/Akt to enhance survival and Rac to induce migration (Taha et al., 2004; Pebay et al., 2007). $S1P_2$ and $S1P_3$ are also widely expressed, and couple to G_i, G_q and G_{12}. Both of these receptors activate ERK1/2 and PLC. While $S1P_2$ also activates Ras, it inhibits Rac, promoting stress fibre formation and inhibiting cell migration. This is quite different to $S1P_3$ which activates Ras, Rac and Rho, resulting in the cell undergoing migratory responses. Unlike the other S1P receptors, $S1P_4$ and $S1P_5$ have a more restricted expression. $S1P_4$ expression appears restricted to hematopoietic lineages and couples to G_i and G_{12} proteins. These activate cell division cycle 42 (Cdc42), PLC, ERK1/2, Rac and adenylate cyclase (AC). In addition, activation of Rho enhances cytoskeletal changes. Similar to $S1P_4$, $S1P_5$ couples to G_i and G_{12} proteins, but engagement of this receptor acts antagonistically to other S1P receptors, as it inhibits AC and ERK1/2 activation, to decrease cell growth (reviewed in Taha et al., 2004; Pebay et al., 2007). Although there is redundancy between the downstream effectors of the S1P receptors, differential responses are activated by engagement of each of these receptors. This is thought to be mediated by distinguishable interactions with extracellular S1P, in addition to variations in signalling strengths through intracellular second messengers (reviewed in Taha et al., 2004).

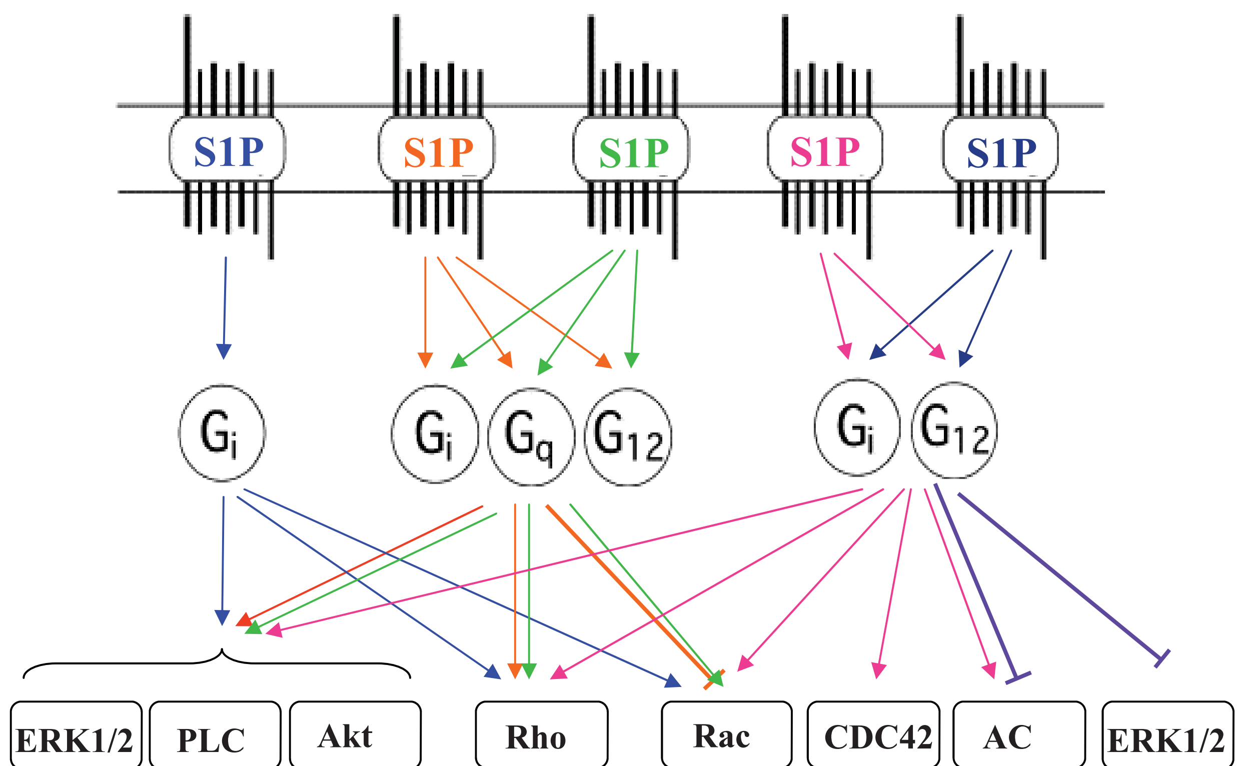

Figure 1.3. G protein-based pathways of transduction utilised by S1P receptors. S1P secreted from cells becomes a ligand for a family of five G protein-coupled receptors on the cell surface. The various S1P receptors couple to different G proteins, regulating numerous downstream signalling pathways. These include signalling through the ERK, adenylate cyclase, phospholipases C, and the PI-3/Akt pathway. Hence, differential expression of the various S1P receptors and their coupling allows extracellular S1P to regulate diverse physiological processes including cell migration, angiogenesis, cytoskeletal organization and differentiation in a highly cell-specific manner.

In addition to this extracellular role, intracellular targets of S1P have long been proposed, although the identities of these remain largely unknown. This evidence was provided by microinjection studies, whereby microinjected S1P was able to mobilize intracellular calcium stores in cells pretreated with pertussis toxin to inactivate their G-coupled receptor signalling. Subsequently, microinjected S1P both induced proliferation in NIH3T3 fibroblasts in a G-protein coupled receptor independent manner, and inhibited apoptosis of mouse oocytes triggered by the anti-tumour drug doxorubicin (Olivera and Spiegel, 2001). Furthermore, overexpression of SK1 in cells lacking functional S1P receptors was still able to stimulate growth (Olivera et al., 2003). Hence it has been proposed that the intracellular signalling of S1P is responsible for the enhanced proliferation, and suppressed apoptosis observed upon activation of SK1 (Figure 1.4). Only recently have several direct intracellular targets of S1P been identified. Maceyka and colleagues (2008) identified PAK1 as being directly activated by low concentrations of S1P, but not sphingosine using *in vitro* kinase assays. These authors suggest that PAK1 activation may be mediated by dual actions of S1P; both intracellularly and by 'inside-out signalling' through $S1P_1$ and its downstream target RAC, to promote S1P mediated cytoskeletal rearrangement and motility (Maceyka et al., 2008). More recently, histone acetylation in the nucleus and subsequent epigenetic gene regulation was proposed to by regulated by S1P through its direct association with histone deacetylases (Hait et al., 2009). SK2 in the nucleus generates S1P from the large nuclear sphingosine pool. Nuclear S1P was shown to interact directly with HDAC1 and HDAC2, with S1P inhibiting the enzymatic activity of these histone deactetylases and preventing the removal of acetyl groups from lysine residues within histone tails. SK2 was also shown to associate with HDAC1 and HDAC2 in repressor complexes, with enrichment of these complexes found at the promoter regions of genes encoding p21 and c-fos, enhancing histone H3 acetylation and local gene transcription (Hait et al., 2009). Hence, these studies provide evidence for a role of S1P in epigenetic gene regulation.

One of the many outcomes of SK1 activation, and subsequent S1P production is an increase in cytosolic Ca^{2+} levels. The mechanism for this SK1-induced Ca^{2+} flux is still the subject of much contention, with evidence favouring the involvement of several different pathways for this occurrence. One of the proposed mechanisms for the increase in cytosolic Ca^{2+} is through the store-operated calcium release-activated calcium current (ICRAC). This plasma membrane channel, important for replenishing Ca^{2+} stores, is inhibited by sphingosine

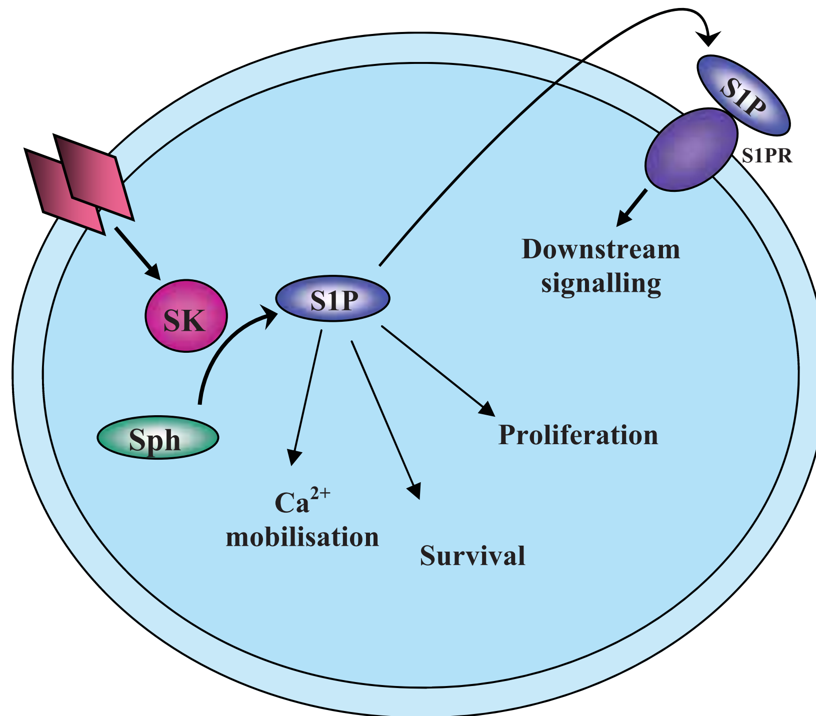

Figure 1.4. S1P is thought to have dual actions, potentially acting both inside and outside of cells.

Inside the cell, activation of SK1 results in an increase in cytoplasmic S1P, with a number of different outcomes including cell growth, survival and Ca^{2+} mobilisation. In addition, S1P can be secreted from the cell and act either in an autocrine or paracrine fashion, becoming a ligand for a family of five G protein-coupled receptors on the cell surface.

(Mathes et al., 1998). Hence, the activation of SK1 and subsequent depletion of cellular sphingosine levels results in relinquishment of this inhibition, thereby increasing calcium influx. Interestingly, TRPC5, a member of the transient receptor potential (TRP) proteins channel is stimulated by both intracellular and extracellular S1P (Xu et al., 2006). These ion channels are permeable to Ca^{2+}, K^{+} and Na^{+} and appear to have a multiplicity of modulators, activators and inhibitors (Beech et al., 2009). S1P applied to cells expressing TRPC5 stimulates this channel, elevating intracellular Ca^{2+} levels (Xu et al., 2006). Blockage of this ion channel inhibited S1P induced motility, suggesting Ca^{2+} influx through this channel is critical for this S1P mediated event (Xu et al., 2006). In addition, the generation of S1P has been proposed to result in the direct release of Ca^{2+} from the ER, however the targets (S1P receptors) of S1P on the ER have yet to be identified. Although evidence suggests that S1P can mobilise Ca^{2+} in an inositol trisphosphate-independent manner, one must consider the possible engagement of S1P receptors on the cell surface. Several S1P receptors are coupled to the activation of PLC, leading to inositol triphosphate formation and hence, Ca^{2+} may be mobilised from the ER in this manner (reviewed in Spiegel and Milstien, 2003b) (Figure 1.5). S1P has also been shown to regulate Ca^{2+} levels in yeast and in higher plants, despite these organisms not having S1P receptors (Spiegel and Milstien, 2003b). Hence, S1P may have a widely conserved function in the regulation of cellular Ca^{2+} levels.

1.5 SK1 and S1P in Platelet Aggregation

In addition to red blood cells and vascular endothelial cells, platelets are one of the major contributors to S1P levels in the plasma (reviewed in Yatomi, 2008). These anucleate cells store abundant levels of S1P, due to a combination of their high SK activity (Buehrer and Bell, 1992; Stoffel et al., 1973a; Stoffel et al., 1973b), and lack of S1P lyase (Stoffel et al., 1973a; Yatomi et al., 1997). Consequently, S1P accumulates in these cells, to be released into the plasma upon stimulation with physiological agonists such as thrombin (Yatomi et al., 1997). This thrombin-mediated release of S1P from activated platelets has been identified as mediated through an ABC transporter (Kobayashi et al., 2006). Once in the extracellular environment, it has been suggested that S1P can further activate neighbouring platelets, leading to platelet aggregation (Yatomi, 2008). Evidence for this has come from studies whereby exogenously added S1P has induced platelet aggregation (Yatomi et al.,

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of the print copy of the thesis held in
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(Spiegel and Milstein, 2003b)

Figure 1.5. Proposed models for Ca^{2+} mobilisation upon activation of SK1.
The store-operated calcium release-activated calcium current (ICRAC) is inhibited by sphingosine. Hence, activation of SK1 leading to depletion of cellular sphingosine levels results disinhibition of this channel, increasing calcium influx. Alternatively, S1P may directly release Ca^{2+} from the endoplasmic reticulum (ER), however the targets (S1P receptors) of S1P on the ER have not yet been identified. In addition, engagement of specific S1P receptors on the plasma membrane can activate of phospholipase C, leading to inositol trisphosphate formation and mobilising Ca^{2+} from the ER.

1995a). In addition, S1P addition induced intracellular Ca^{2+} mobilisation, with the dose response for Ca^{2+} release closely correlated with that required for shape change (Yatomi et al., 1995a). Hence S1P is suggested to be an paracrine stimulator of platelet activation and has a likely role in platelet-mediated wound repair and the maintenance of vascular integrity (Yatomi, 2008).

Although platelets are known to possess high SK activity, and S1P appears to function in platelet aggregation, the direct role of SK1 in platelet aggregation has yet to be clarified. Yatomi and colleagues (1996) were able to demonstrate a role for SK1 in thrombin receptor activator protein (TRAP)-induced platelet activation, with the SK1 inhibitor, *N,N*-dimethylsphingosine (DMS), preventing platelet aggregation. However later studies have shown no overall change in platelet SK1 activity upon activation with a wide range of agonists including thrombin, collagen, and adrenaline, despite these stimulators strongly inducing platelet activation (Yatomi et al., 1997; Ohmori et al., 2005). However, it is possible that SK1 may influence platelet activation through a change in subcellular localisation rather than an overall increase in activity. Ohmori and colleagues were able to demonstrate an increase in SK1 activity in the membrane fraction of platelets when specifically activated through the collagen receptor (Ohmori et al., 2005). In addition, Ca^{2+} mobilisation specifically induced by collagen or convulxin (an agonist of the collagen receptor) was inhibited by the use of DMS in platelets but this was not seen with G-protein stimulating agonists such as ADP, TRAP or thrombin (Ohmori et al., 2005; Yang et al., 1999). Hence, it appears that SK1 activity may be specifically involved in collagen-induced platelet activation and Ca^{2+} mobilisation through a change in this enzyme's subcellular localisation.

1.6 SK1 and Disease

Elevated levels of SK1 have been implicated in a number of different diseases, including asthma, inflammation and atherosclerosis, and cancer (see below).

Inflammation

There is substantial evidence now linking SK1 with inflammation and immunity (reviewed in Melendez, 2008b). S1P receptors have been demonstrated to be critical for lymphocyte egress from the lymph organs, enabling their access to inflammatory lesions (Brinkmann et al., 2001). SK1 has also been shown to regulate neutrophil priming, to provide a critical defence against infections (MacKinnon et al., 2002). In addition, SK1 is involved neutrophil activation, including chemotaxis (Ibrahim et al., 2004). In macrophages, SK1 activity has been shown to be activated through a wide range of stimuli, with the subsequent generation of S1P reported to advance wound-healing processes *in vitro* and *in vivo* (English et al., 2001), in a macrophage dependent manner. SK1 activity has also been shown to modulate the expression of adhesion molecules in vascular endothelial cells, essential for the recruitment of leukocytes during inflammatory responses (Baumruker and Prieschl, 2002; Xia et al., 1998). This proposed role of SK1 in inflammation has, however become contentious with more a recent study demonstrating unaffected inflammatory responses in SK1 knock-out mice (Michaud et al., 2006). In this study, SK1 appeared dispensable in both acute inflammatory responses (in thioglycollate-induced peritonitis) and chronic inflammation using the collagen-induced model of rheumatoid arthritis (Michaud et al., 2006). Hence, more extensive studies on the requirement of SK1 in inflammation are necessary.

Asthma

SK1 in asthma appears to determine the allergic responsiveness of mast cells (Melendez, 2008a), with SK1 critical for calcium responses and degranulation, triggered by FcεRI (Melendez and Khaw, 2002). In addition, some proinflammatory molecules, including TNF-α, secreted from mast cells following FcεRI stimulation, are completely inhibited by high intracellular levels of sphingosine (Prieschl et al., 1999). Thus, SK1 activity is thought to not only generate a second messenger (S1P), but, through its reduction in sphingosine levels, also remove an inhibitory one (Melendez, 2008a). Hence, the sphingolipid rheostat has been proposed to be involved in the fine tuning of allergic susceptibility of mast cells by FcεRI engagement (Prieschl et al., 1999). The S1P generated following FcεRI stimulation

and SK1 activation is secreted from the cell, then acting through its receptors $S1P_1$ and $S1P_2$ to amplify the responses observed during FcεRI activation (Jolly et al., 2004).

Of interest, elevated levels of S1P have been recovered from the broncho-alveolar lavage (BAL) fluid from allergic asthma patients after antigen challenge (Ammit et al., 2001; Rosenfeldt et al., 2003). This has been suggested to play a role in both acute broncho-constriction and airway remodelling through its direct action on airway smooth muscle cells. Furthermore, it has recently been shown that siRNA silencing of SK1 *in vivo* protects mice from allergic asthma induced by ovalbumin (OVA) immunisation (Lai et al., 2008). In addition, inhalation of SK1 inhibitors following OVA-challenge decreased S1P levels present in BAL fluid in mice, as well as reducing eosinophil infiltration and peroxidase activity (Nishiuma et al., 2008). Bronchial hyper responsiveness induced by inhaled methacholine and goblet cell hyperplasia were also improved by SK inhibitors in this study (Nishiuma et al., 2008).

Atherosclerosis

SK1 activation has also been implicated in the pathogenesis of atherosclerosis. This involvement arises from several different observations. S1P in the serum is complexed to plasma lipoproteins HDL and LDL. In addition to it being a major risk factor for atherosclerosis, oxidized LDL can sequentially induce sphingomyelinase, ceramidase and SK1 in smooth muscle cells (Auge et al., 1999). This results in S1P production and enhanced mitogenesis of these cells. In endothelial cells, SK1 activation is involved in the TNF-α induced upregulation of adhesion molecules E-selectin and vascular cell adhesion molecule (VCAM) (Xia et al., 1998). HDL interrupts the activation of SK1 following TNF-α stimulation, inhibiting these effects (Xia et al., 1999a). This supports an anti-atherogenic role for HDL via inhibition of intracellular SK activation and S1P production by proinflammatory cytokines. Clinical data has also shown that S1P is more predictive of obstructive coronary artery disease than other well established risk factors (Deutschman et al., 2003). The levels of serum S1P also correlated with the severity of the disease (Deutschman et al., 2003). These studies, therefore, point to an active role for SK1 and S1P in atherogenesis, with some studies implicating the lipid as a mediator of atherosclerosis.

Cancer

In addition to roles in each of these diseases, the most studied patho-physiological role of SK1 by far is in cancer. The initial clues for a potential role of SK1 in oncogenesis came from the previously described studies whereby overexpression of SK1 resulted in decreased apoptosis and increased proliferation in a number of cultured cell lines (Olivera et al., 1999; Maceyka et al., 2002; Xia et al., 2000; Pitson et al., 2005). Subsequently, it was shown that overexpression of SK1 in NIH3T3 fibroblasts resulted in neoplastic transformation and the ability to form tumours in mice (Xia et al., 2000). Additionally, SK1 activity has been implicated in the transforming ability of the well-known oncogene H-Ras, with the use of SK1 inhibitors or the expression of inactive SK1 that blocks SK1 activation able to block Ras-induced transformation *in vitro* (Xia et al., 2000). Furthermore, accumulating evidence by a number of different studies supports a role of deregulation of SK1 in naturally occurring tumours. These include the identification of increased SK1 mRNA and protein in a variety of human tumours (French et al., 2003; Van, Jr. et al., 2005; Li et al., 2008; Bayerl et al., 2008; Kawamori et al., 2006; Kawamori et al., 2009), a correlation between high SK1 expression and poor prognosis in human glioblastoma, gastric cancer and breast cancer patients (Van, Jr. et al., 2005; Li et al., 2008; Ruckhaberle et al., 2008) and a critical requirement of SK1 in estrogen-dependent regulation of breast tumour cell growth and survival (Sukocheva et al., 2003). In addition, colon cancers with metastasis had a higher expression of SK1 than those without metastasis (Kawamori et al., 2009). Angiogenic factors and processes are also affected by SK1 and S1P, including cell motility, mitogenesis in smooth muscle cells, endothelial cell differentiation, and growth factor signalling, providing further evidence of SK1 representing an attractive target in cancer therapeutics (reviewed in French et al., 2006).

Additionally, considerable evidence now indicates a deregulation of SK1 in both acute and chronic myeloid leukaemia. Elevated levels of SK1 have been identified in a variety of leukaemic cell lines, correlating with chemotherapeutic resistance (Sobue et al., 2008), while high SK1 expression appears to be integral for erythroleukaemic progression (Le et al., 2005). In addition, SK1 expression has been shown to be upregulated by the BCR/ABL gene fusion, with this event necessary for Mcl-1 expression and enhanced cell survival in chronic myeloid leukaemia (CML) (Li et al., 2007).

Encouragingly, the targeting of SK1 activity has shown promising results in the treatment of cancers *in vitro* and *in vivo*. This was demonstrated by the use of a dominant-negative SK1 mutant and siRNA-mediated suppression of SK1 expression to sensitise tumour cells to chemotherapeutics (French et al., 2006). Also compellingly, the use of SK1 inhibitors was able to significantly reduce tumour growth *in vivo* in mice (French et al., 2003), while the genetic ablation of SK1 significantly reduced tumour growth in both a mouse model of intestinal adenoma (Kohno et al., 2006) as well as the azoxymethane (AOM)-induced murine model of colon cancer (Kawamori et al., 2009). In addition, siRNA mediated suppression of SK1, or the targeting of this enzyme by chemical inhibitors induces apoptosis in acute myeloid leukaemia (AML) cells (Paugh et al., 2008), and alleviates chemotherapeutic resistance in both AML and CML cells (Baran et al., 2007; Bonhoure et al., 2005; Bonhoure et al., 2008; Ricci et al., 2009). Hence, SK1 appears to be a promising target for the treatment of a wide variety of cancers.

Although the mechanism by which SK1 exerts its oncogenic effects are not yet known, studies have indicated that this is independent of G protein-coupled receptors, mediated exclusively by intracellular S1P generated by SK1 activity (Olivera et al., 2003). Consequently, much recent work has focused on the intracellular mechanism(s) by which SK1 activity exerts this oncogenic phenotype in cells.

1.7 Activation of SK1 in Cells

As previously described, SK1 possesses intrinsic catalytic activity that is not dependent on post-translational modification (Pitson et al., 2000a). This basal activity of SK1 has been proposed to be involved in a house-keeping role of the enzyme, in clearing sphingosine and upstream lipids from the cell (Wattenberg et al., 2006; Leclercq and Pitson, 2006). The catalytic activity of SK1, however, can be transiently activated by a number of different agonists including, among others, TNF-α (Xia et al., 1998), interleukin-1β (Mastrandrea et al., 2005), platelet-derived growth factor (PDGF) (Olivera and Spiegel, 1993), vascular endothelial growth factor (VEGF) (Shu et al., 2002), epithelial growth factor (EGF) (Meyer zu et al., 1999), nerve growth factor (NGF) (Rius et al., 1997), and surprisingly sphingosine-1-phosphate itself (Meyer zu et al., 2001). While the magnitude of this activation is generally modest (approximately 2-fold), it does result in an increase in cellular and

secreted S1P (Pitson et al., 2000a; Pitson et al., 2003). This activation of SK1 appears the result of phosphorylation at residue Ser225, with mutation of this residue to alanine completely blocking the TNF-α or protein kinase C (PKC) induced increase in catalytic activity, while not affecting the constitutive basal activity of the enzyme (Pitson et al., 2003). Furthermore, this phosphorylation of SK1 is mediated by ERK1/2 and results in a direct 14-fold increase in the k_{cat} for the enzyme, while having minimal effect on the K_M for either substrate (Pitson et al., 2003).

1.8 SK1 Activation and Oncogenic Signalling

SK1 activation has a well-described survival phenotype, with enhanced S1P levels able to protect cells from serum-deprivation-, ceramide- and TNF-α-induced apoptosis (Maceyka et al., 2002; Pitson et al., 2005; Xia et al., 2000). These anti-apoptotic effects of SK1 can be partly attributed to the lowering of sphingosine and ceramide levels in the cell. They may also be partly due to signalling through the SIP receptors and currently undefined intracellular roles of S1P. In addition, Xia and colleagues identified a tumour necrosis factor receptor-associated factor 2 (TRAF2)-binding motif in SK1. This motif mediated an interaction between TRAF2 and SK1, which was necessary for the activation of SK1 in response to TNF-α. This SK1 activity was required for TNF-α -mediated activation of NF-κB and antiapoptotic signalling by this cytokine (Xia et al., 2002) (Figure 1.6).

In addition to this anti-apoptotic role, overexpression of SK1 is known to enhance cell proliferation. The importance of SK1 phosphorylation at Ser225 and subsequent activation in its cellular signalling roles has been established since a non-phosphorylatable mutant of this enzyme is unable to support the enhanced growth and survival found in cells overexpressing *wild-type* SK1 (Pitson et al., 2005). Similarly, the importance of this phosphorylation site for the oncogenic effects of this enzyme has also been established. As described earlier, cells overexpressing *wild-type* SK1 undergo neoplastic transformation as determined by colony growth in soft agar, focus formation and tumour formation in mice. Ablation of the Ser225 phosphorylation site in SK1 through mutagenesis (Ser→Ala), however substantially reduced this transformation. Interestingly, the SK1 Ser225 mutant retained full intrinsic catalytic activity, suggesting this phosphorylation influenced the

NOTE:
This figure is included on page 15a of the print copy of the thesis held in the University of Adelaide Library.

(Xia et al., 2002)

Figure 1.6. Model for the SK1-mediated anti-apoptotic signalling through the TNF-α receptor.

Upon engagement of the TNF-α receptor, SK1 interaction with TRAF2 increases SK1 activity. This is required for NF-κB (but not JNK) activation, and subsequent anti-apoptotic signalling.

oncogenic signalling of SK1 in a manner independent of the increase in catalytic activity (Pitson et al., 2005).

1.9 SK1 Translocation

In addition to an increase in catalytic activity, numerous studies have shown that activation of SK1 in response to agonist stimulation results in a translocation of SK1 from the cytoplasm to the plasma membrane. This translocation has been shown to result from physiological stimuli including PDGF (Rosenfeldt et al., 2001), NGF (Toman et al., 2004) and insulin-like growth factor (El-Shewy et al., 2006), TNF-α (Pitson et al., 2005), IgE (Jolly et al., 2004), and lysophosphatidic acid (LPA) (Delon et al., 2004). The translocation of this enzyme has also been observed upon Ca^{2+} mobilisation (Young et al., 2003) and phorbol ester stimulation (Johnson et al., 2002; Pitson et al., 2003). Interestingly, Pitson and colleagues were able ablate the phorbol myristoyl acetate (PMA) and TNF-α induced translocation of SK1, both by mutation of the SK1 phosphorylation site, as well as the use of an ERK1/2 pathway inhibitor U0126 (Pitson et al., 2003), hence demonstrating the importance of this ERK1/2 mediated phosphorylation of SK1 in the localisation of the enzyme. To investigate whether the localisation of SK1 was important for its oncogenic signalling, non-phosphorylatable SK1 was constitutively localized to the plasma membrane via attachment of the Lck tyrosine kinase myristoylation/ dual palmitoylation motif. This artificial localisation of the non-phosphorylatable SK1 was sufficient to restore its ability to transform cells (Pitson et al., 2005). Together, these findings suggest a model whereby activation of ERK1/2 phosphorylates SK1 at Ser225, both increasing the catalytic activity of this enzyme and resulting in its translocation to the plasma membrane, with these events required for the downstream oncogenic signalling of SK1 (Figure 1.7).

1.10 Translocation Mechanism of SK1 to the Plasma Membrane

The previously mentioned translocation of SK1 to the plasma membrane is hypothesized to function to bring the enzyme into close proximity with its substrate. Both sphingosine and dihydrosphingosine, the lipid substrates of SK are largely generated at the plasma membrane (Khan et al., 1991), and thereby targeting SK to this site would logically increase

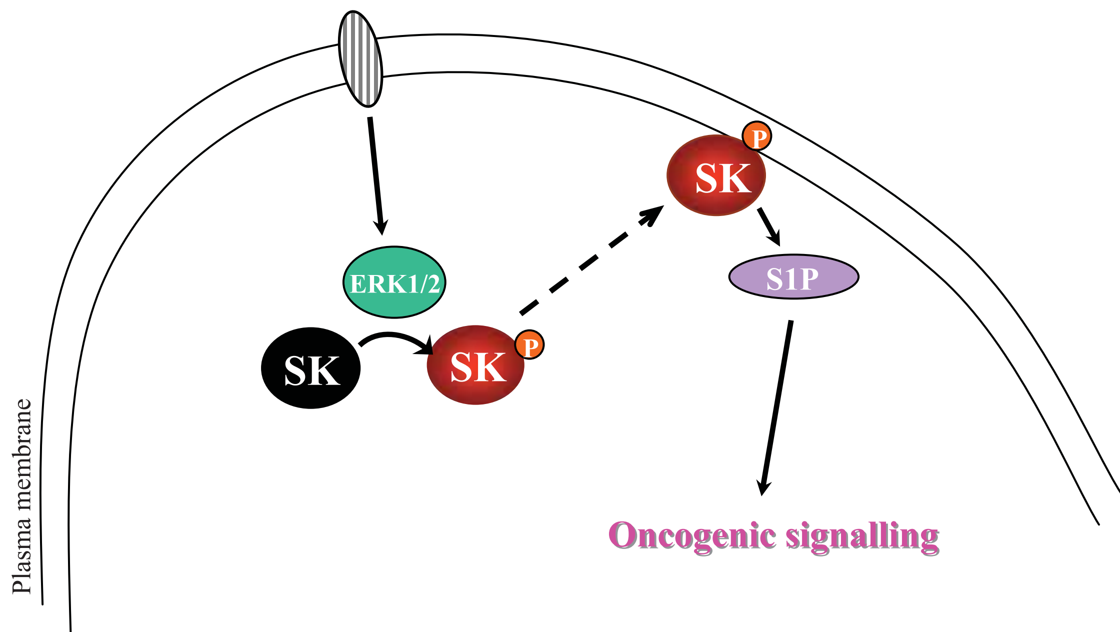

Figure 1.7. Mechanism for SK1 oncogenic signalling.

Following agonist stimulation, ERK1/2 phosphorylates SK1 at Ser^{225}, resulting in both an increase in enzyme activity and its subsequent translocation from the cytosol to the plasma membrane. Both of these events are critical in the downstream oncogenic signalling by SK1.

the levels of S1P produced resulting in enhanced S1P release or intracellular signalling. However, the mechanism by which the phosphorylation of SK1 is able to direct its translocation to the plasma membrane is currently unknown. Delon and colleagues demonstrated SK1 to have a high affinity for phosphatidic acid (PA), with SK1 also translocating to PA-enriched regions of the plasma membrane upon phorbol ester stimulation (Delon et al., 2004). Hence, a role for this phospholipid in the plasma membrane targeting of SK1 was speculated. In a subsequent study, however, SK1 was shown to have a five-fold higher affinity for phosphatidyl serine (PS) than PA (Stahelin et al., 2005). This suggested the involvement of PS, rather than PA in the localisation of SK1 to membranes. In addition, a preferential interaction between phosphorylated SK1 and PS-containing model membranes was demonstrated *in vitro*. Mutations in SK1 (including Ser225 → Ala) which blocked the association of the enzyme with model membranes *in vitro*, also prevented the translocation of SK1 to the membrane in HEK293 cells. Based on these experiments, the investigators proposed that upon phosphorylation of SK1, a PS binding site in the enzyme becomes exposed, driving the translocation of SK1 to the plasma membrane. However, as the majority of this study was performed *in vitro* with model membranes, the validity of a PS-mediated translocation of SK1 *in vivo* remains uncertain. While the observations by Stahelin and colleagues may not completely expound the translocation of SK1, they do suggest a possible model whereby upon translocation, phosphorylated SK1 may be retained at the plasma membrane by interaction with PS, increasing its probability of contacting and acting on its substrate sphingosine.

In addition to the above mentioned experiments, Maceyka and colleagues have recently proposed a role for Filamin A (FLNa) in the translocation of this enzyme (Maceyka et al., 2008). FLNa is an actin binding protein that cross-links cortical actin filaments into dynamic actin webs at membrane ruffles (Stossel et al., 2001). Maceyka and colleagues (2008) were able to demonstrate an endogenous interaction between FLNa and SK1 in mammalian cells, and show a colocalisation of these two proteins at the lamellipodia in A7 melanoma cells upon stimulation with Heregulin (Hrg), an EGF-like ligand. As SK1 localisation to the lamellipodia was not observed in parental M2 melanoma cells lacking FLNa, and ectopic expression of FLNa in these cells restored both the Hrg-induced formation of membrane ruffles and colocalisation of FLNa and SphK1 at the lamellipodia, these authors suggested a direct requirement for FLNa in the Hrg-induced translocation of SK1 (Maceyka et al., 2008). This lamellipodial localisation of SK1 did not appear to be

dependent upon ERK1/2 mediated phosphorylation, as ERK1/2 phosphorylation was intact in parental M2 melanoma cells lacking FLNa. These authors further showed an FLNa dependent colocalisation of SK1, FLNa and $S1P_1$, but not $S1P_2$, at the lamellipodia upon Hrg stimulation, suggesting that FLNa links SK1 to this S1P receptor (Maceyka et al., 2008). Although this study certainly identifies a role for FLNa in the lamellipodia localisation of SK1, it is possible the role of this actin-binding protein could be to simply link SK1 with its S1P receptor at the cell membrane, rather than actively transport it to this location. Also, as ERK1/2 mediated SK1 phosphorylation did not seem to play a role in the translocation of this enzyme in this system, multiple translocation mechanisms may exist which vary dependent upon cell type and the SK1 activating agonist.

In addition to the previously mentioned experiments, Young et al. (2003) have suggested a direct role for the Ca^{2+} activated protein calmodulin (CaM) in the translocation of SK1. This hypothesis stems from experiments whereby the CaM inhibitor W7, was demonstrated to inhibit the translocation of SK1 from the cytosol to the plasma membrane in response to Ca^{2+} mobilising stimuli. Numerous studies have shown the ability of CaM to interact with SK1 in a Ca^{2+}-dependent manner *in vitro* (Pitson et al., 2000b; Olivera et al., 1998; Pitson et al., 2001; Pitson et al., 2002). Hence this evidence, combined with its common regulatory role in controlling the activity of a number of signalling enzymes (Tanaka, 1988), has led to a role for CaM in SK1 regulation being speculated (Olivera and Spiegel, 2001; Liu et al., 2002). This hypothesis gained further strength following studies that identified residues in SK1 critical for CaM binding (Sutherland et al., 2006).

To investigate the potential involvement of CaM in the agonist-dependent translocation of SK1 to the plasma membrane, both *wild type* SK1 and SK1 with its CaM binding site mutated ($SK1^{F197A/L198Q}$) were expressed in HEK293T cells as fusion proteins with enhanced green fluorescent protein (EGFP), and their localisation examined by both immunofluorescence and subcellular fractionation upon exposure of the cells to PMA. In stark contrast to the redistribution of the enzyme to the plasma membrane observed with *wild-type* SK1 upon this treatment, no redistribution of $SK1^{F197A/L198Q}$ was observed in response to PMA (Sutherland et al., 2006). It is important to note that this mutant of SK1 is still capable of being phosphorylated and retains high activity (Sutherland et al., 2006). Thus, these results indicate a role for the CaM binding site of SK1 in its Ca^{2+}-dependent

translocation to the plasma membrane. However, despite these studies, a direct role for CaM itself in mediating the translocation of this enzyme is yet to be established.

In addition to the lack of direct evidence for a role for CaM in mediating SK1 translocation to the plasma membrane, other anomalies still exist within this model, questioning the likelihood of CaM acting as the shuttle for SK1. Firstly is the absence of an obvious targeting motif that could function to direct CaM to the plasma membrane. Secondly, although Ca^{2+}-dependent translocation of CaM in cells has been observed previously, the main destination for this redistribution appears to be the nucleus, rather than the plasma membrane (Chin and Means, 2000; Pruschy et al., 1994; Deisseroth et al., 1998). Lastly, the inability to co-immunoprecipitate SK1 with CaM from cells, even following overexpression of both proteins (Pitson S.M., unpublished data), questions the ability of these two proteins to interact in cells. Consequently, whether the protein responsible for the translocation of SK1 is actually CaM or a CaM-like molecule remains to be determined.

1.11 CIB1, a CaM-like Molecule, also Interacts with SK1

In an attempt to identify SK1 interacting proteins, the Pitson laboratory performed a yeast-two-hybrid screen using full length human SK1 as bait and utilizing a human leukocyte cDNA library (Zebol et al., 2009; Leclercq et al., 2008). One putative SK1-interacting protein identified in this screen was calcium-and-integrin binding protein 1 (CIB1), also known as kinase interacting protein (KIP) and calmyrin. Since CIB1, and its related proteins are a major focus of this thesis, they are reviewed in detail below.

1.11.1 CIB1

CIB1 was first identified as interacting in a Ca^{2+}-dependent manner with the cytoplasmic domain of the platelet specific αIIb integrin (Naik et al., 1997). In this complex, CIB1 has a role in platelet activation and aggregation (see below). However, further studies have since identified multiple binding partners of CIB1, with wide-ranging regulatory roles in the cell speculated, as described below. CIB1 is a four EF-hand containing protein most similar in amino acid sequence to calcineurin B (58%) (Gentry et al., 2005). Interestingly, CIB1 also

shares 55% amino acid similarity with CaM. Thus, this raised the possibility that perhaps CIB1, and not CaM, mediates the translocation of SK1 to the plasma membrane.

CIB1 also appears structurally similar to the neuronal calcium sensor (NSC) family of proteins. These proteins are such termed due to their neuronal expression pattern, ability to bind Ca^{2+} and sequence homology (Braunewell and Gundelfinger, 1999). This family typically contains four EF-hand domains, enabling the proteins to bind Ca^{2+} at levels just above basal Ca^{2+} concentrations, with typically a 10-fold higher affinity than that of CaM (Gentry et al., 2005). The NCS proteins have a variety of functions. One of these proteins, recoverin, directly inhibits rhodopsin kinase (Senin et al., 2002), while other NCS proteins, including frequenin modulate synaptic activity, neurotransmitter release and vesicle secretion (Braunewell and Gundelfinger, 1999). The NCS proteins typically have a common mode of action, enabling cells to respond to very small changes in intracellular Ca^{2+} concentration by binding Ca^{2+} into one or more of their EF-hand domains. This binding of Ca^{2+} results in the protein undergoing a conformational change, enabling it to interact with target proteins (Ames et al., 1997; Braunewell and Gundelfinger, 1999). Unlike its NCS homologs, CIB1 expression is not restricted to the neurone, and has been shown to be expressed in a wide range of human tissues (Shock et al., 1999). This suggests a more global regulatory role of CIB1 may be possible.

1.11.2 Structural Characterisation of CIB1

Numerous structural studies have shown CIB1 to contain four EF-hand domains, termed EF1-EF4, the last two of which have been implicated in Ca^{2+} binding (Yamniuk et al., 2004; Gentry et al., 2005; Yamniuk and Vogel, 2005; Blamey et al., 2005; Hwang and Vogel, 2000). Although several of its related proteins, including KChIP3 (Osawa et al., 2001), and neurocalcin (Vijay-Kumar and Kumar, 1999) have been shown to function as dimers, Ca^{2+}-CIB1 crystallized as a monomer, with CIB1 also monomeric in solution as revealed by diffusion NMR spectroscopy (Weljie et al., 2003), sedimentation equilibrium and gel filtration studies (Gentry et al., 2005; Sobczak et al., 2005). Importantly, X-ray crystal structures have shown CIB1 to undergo significant structural changes upon binding to Ca^{2+}. Yamniuk et al. (2004) found that in the absence of any bound metal ions, apo-CIB1 adopts a folded yet highly flexible molten globule-like structure. However, the binding of Ca^{2+}

induced a conformational change which stabilized both the secondary and tertiary structure of CIB1, and substantially increased the thermal stability of the protein. Like its homologs, Ca^{2+}-bound CIB1 folds into closely associated globular N- and C-terminal domains separated by a flexible linker, each containing two EF-hands, with extended N- and C-terminal regions (Yamniuk et al., 2004). Ca^{2+} binds to the two C-terminal EF-hands, EF3 and EF4 in a sequential manner with affinities of 1.9 and 0.54 μM respectively (Yamniuk et al., 2004). These affinities are comparable to those seen in NCS proteins and enabling the binding of Ca^{2+} at concentrations just above basal levels (Yamniuk et al., 2004). The sequences of the two N-terminal EF-hands are quite divergent, lacking many of the required conserved residues and hence remain Ca^{2+} free (Gentry et al., 2005) (Figure 1.8A).

Interestingly, EF3 of CIB1 has also been shown to be a low-affinity Mg^{2+} binding site, with Mg^{2+} binding eliciting similar structural conformational changes in CIB1 as that of Ca^{2+} (Yamniuk et al., 2004). Mg^{2+} binding was able to substitute for Ca^{2+} and enable the interaction of CIB1 with αIIb integrin cytoplasmic domain peptides *in vitro*, albeit through a thermodynamically distinct manner (Yamniuk and Vogel, 2005). This raises the question of whether Mg^{2+} may also play a wider regulatory role in contributing to the activity of this protein *in vivo*. Notably, subtle structural differences between Mg^{2+}- and Ca^{2+}-bound CIB1 have been revealed by NMR spectroscopy, suggesting the possibility that Mg^{2+} may facilitate interactions of CIB1 with only a subset of its binding partners. While Mg^{2+} binds to EF3 with a low affinity of 120 μM, this metal ion is present in millimolar concentrations inside the cell. This has led to suggestions that under basal conditions, EF3 is constitutively occupied by Mg^{2+} (Yamniuk et al., 2004; Yamniuk and Vogel, 2005). Increased cytosolic Ca^{2+} levels are predicted to cause the low affinity binding of Mg^{2+} to be displaced by its higher affinity association for Ca^{2+}. As such, the basal binding of Mg^{2+} may enable CIB1 to adopt a structure suitable for facilitating interaction with some of its binding partners, independent of Ca^{2+}.

Barry et al. (2002) characterized the molecular basis of CIB1 binding to the integrin αIIb, with the CIB1 binding site residing within a hydrophobic, 15 amino acid membrane-proximal region of αIIb (Barry et al., 2002). Interestingly, this region is analogous to the α-helical targets of other EF-hand proteins, including CaM. Subsequently, the corresponding interacting region on CIB1 was revealed by NMR and chemical shift perturbation mapping as a hydrophobic channel spanning the entire C- and part of the N-terminal domain

A

NOTE:
These figures are included on page 21a of the print copy of the thesis held in the University of Adelaide Library.

(Gentry et al., 2005)

B

(Gentry et al., 2005)

Figure 1.8. Structural characterisation of CIB1

(A) Ribbon structure of Ca^{2+}-bound CIB1. CIB1 is composed of 4 closely associated EF hands, EF1-EF4. The extended N-terminal region and EF1 are shown in blue, EF2 is yellow, EF3 is red, EF4 is green and the C-terminal helix is purple. Each isolated hand is also depicted below the complete structure. Helices are also labeled, with the dotted line indicating the disordered region between helices H7 and H8. The two C-terminal EF-hands each bind one Ca^{2+} ion, as designated by the gold spheres.

(B) Surface structure of CIB1 coloured to represent the spectrum of hydrophobic potential. Highly hydrophobic residues are coloured in red, while highly hydrophilic residues are coloured blue. The orientation of CIB1 in this surface sturucture is very similar to that (A), with the red hydrophobic binding pocket clearly visible.

(Yamniuk et al., 2006) (Figure 1.8B). Notably, the presence of this large hydrophobic binding pocket is conserved in both CaM and calcineurin B (Gentry et al., 2005).

1.11.3 The Function of CIB1 in Platelet Activation

As mentioned above, CIB1 was first described through its association with the cytoplasmic domain of the platelet-specific integrin αIIb (Naik et al., 1997). The integrin αIIbβ3 is the most abundant integrin on the platelet surface, mediating both platelet aggregation and clot retraction (Shock et al., 1999). Upon platelet activation, αIIbβ3 receives intracellular signals (inside-out signalling) causing it to rapidly change in conformation from a low-affinity state to a high affinity state, enabling it to bind soluble ligands, such as fibrinogen. The binding of fibrinogen to this receptor initiates further intracellular signalling in the platelet (outside-in signalling) resulting in processes required for platelet spreading and clot retraction. Although it is known that the integrin cytoplasmic tail is required for platelet activation, how the cytoplasmic tail of αIIb regulates this is unknown.

Since in its initial discovery, the role of CIB1 in platelet activation has been the subject of several studies. Tsuboi (2002) showed that CIB1 activated αIIbβ3 both *in vitro* and in cells, and its direct association with αIIb increased the affinity of this integrin for fibrinogen. In a later study, the function of CIB1 relative to αIIbβ3 was explored through the manipulation of megakaryocytes (platelet precursors), where CIB1 was either overexpressed, or depleted by RNA interference (Yuan et al., 2006b). In direct contrast to the previous study, results implicated CIB1 as an endogenous inhibitor of the agonist-induced activation of αIIbβ3. In a separate study, a CIB1 antibody was used to block CIB1 function with these results demonstrating the interaction between αIIbβ3 and CIB1 to be necessary for proper platelet spreading on immobilised fibrinogen (Naik and Naik, 2003b). Hence, it was hypothesised that CIB1 may be required to convert αIIbβ3 into an active conformation during inside-out signalling.

Regardless of the effect of CIB1 on platelet activation, CIB1 has been shown to associate with αIIbβ3 subsequent to integrin activation. In addition, CIB1 was shown to interact preferentially with active αIIbβ3, with this association necessary for the release of ADP from platelet granules (Naik and Naik, 2003a). Hence, CIB1 may be important for clot

stabilisation. In addition, CIB1 has been shown to recruit the actin polymeriser Wiskott-Aldrich syndrome protein (WASP) to αIIbβ3 post integrin activation and enhance adhesion (Tsuboi et al., 2006). CIB1 has also been identified as binding to focal adhesion kinase (FAK) in platelets adhering to fibrinogen (Naik and Naik, 2003a). FAK signalling leads to activation of Rho family kinases, and stimulation of actin polymerisation, lamellipodium formation and cell migration (reviewed in Yamniuk and Vogel, 2006). Hence, CIB1 may regulate actin cytoskeletal changes and platelet spreading during inside-out signalling (Yamniuk and Vogel, 2006). Additionally, CIB1 has been demonstrated to bind to the Rho family member, Rac3 and this association was found to have a positive affect on αIIbβ3-mediated fibroblast adhesion, and spreading on fibrinogen (Haataja et al., 2002). Interestingly, however, are two contradictory studies using platelets from CIB1 knock-out mice to study the role of CIB1 in hemostasis. Denofrio and colleagues (2008) showed no overt defect in platelet function in CIB1 deficient platelets, although the observed up-regulation of other CIB1 family members was hypothesised as a compensatory mechanism to account for this. In direct contrast, Naik et al. (2009) showed that a lack of CIB1 did, in fact, result in impaired thrombosis. In this study, CIB1 deficient mice had a significantly increased tail bleeding time and had a delayed occlusion time following $FeCl_3$-induced injury of the carotid artery, with unstable thrombus formation (Naik et al., 2009). While inside-out signalling was normal in CIB1 deficient platelets, the absence of CIB1 affected their ability spread on immobilised fibrinogen, but not form filipodia (Naik et al., 2009). CIB1 was suggested to affect outside-in signalling, as reduced tyrosine-phosphorylation of the integrin β_3 subunit was observed in the CIB1 null platelets (Naik et al., 2009). This study clearly defines a role for CIB1 in thrombosis. To address the conflict with the study by Denofrio and colleagues (2008), these authors hypothesised that the lack of any observable defect in the CIB1 null mice in the first study by may have been due a difference in methodology (Naik et al., 2009). Denofrio and colleagues (2008) used a more severe injury in both their tail bleeding and $FeCl_3$ models, and hence, the function of CIB1 in thrombosis may have been masked in these experiments.

1.11.4 Other CIB1 Interacting Proteins

Since its initial discovery in platelets, CIB1 has been shown to interact with a number of different proteins in a wide range of cell types with apparent diverse roles. However as

many of these interactions have only recently been discovered, a detailed analysis of the mechanisms whereby CIB1 can regulate its various target proteins has yet to be performed. However, like the related CaM, initial hints towards the functions of this protein indicate it may have a wide regulatory role in cell signalling pathways.

PAK1

P21-activated kinases (PAKs) are key effectors of Rac1 and Cdc42 and regulate many cellular processes including cell migration, cytoskeletal rearrangement, proliferation, differentiation and gene expression (Kreis and Barnier, 2009; Eswaran et al., 2009). CIB1 was shown to interact with PAK1 in a Ca^{2+} dependent manner, with CIB1 overexpression significantly decreasing cell migration by inducing PAK1 activation (Leisner et al., 2005). Conversely, RNAi mediated depletion of CIB1 increased cell migration and restored normal PAK1 adhesion induced activation (Leisner et al., 2005). Furthermore, PAK1 activation was shown to be dramatically reduced *in vivo* in $CIB1^{-/-}$ ischemic gastrocnemius muscle tissue homogenates (Zayed et al., 2007).

Presenilin 2

Presenilin 2 (PS2) along with PS1 forms an integral membrane component of γ-secretase complexes, responsible for the generation of β-amyloid peptides (Czech et al., 2000). These are quite often characterized as the insoluble senile plaques associated with the pathogenesis of Alzheimer's disease. Deregulation of intracellular Ca^{2+} signalling events is another key event in this disease (Mattson et al., 2000). CIB1 was shown to bind endogenous PS2 specifically (Stabler et al., 1999; Zhu et al., 2004; Blazejczyk et al., 2006), however, quite surprisingly, this association was shown to be Ca^{2+}-independent (Blazejczyk et al., 2006). In addition, the distribution of CIB1 in human forebrain was altered in Alzheimers disease versus normal ageing brains (Bernstein et al., 2005). In normal brain, CIB1 staining was present in pyramidal neurones and interneurones of the palaeo- and neocortex, cerebellar granule cells and hypothalamic neurones of the paraventricular, ventromedial and arcuate nuclei. Moderate CIB1 levels were also present in hippocampal pyramidal cells with stronger staining in dentate gyrus neurones. In Alzheimers disease brain however, a

substantial loss of CIB1 positive neurones was observed in all regions, especially in cortical areas. CIB1 immunosignals were also in part associated with diffuse and senile plaques. Although the CIB1-PS2 interaction has been hypothesized as playing a role in Alzheimer's disease, its function in this context is not yet known.

Pax3

Pax3 is a member of the paired class homeodomain family of transcription factors and is an early marker in myogenic differentiation (Stuart et al., 1994). CIB1 was shown to bind to the region of Pax3 that is involved in DNA binding, and in doing so, inhibits the transcriptional activity of Pax3 (Hollenbach et al., 2002). Hence, CIB1 may impart a potential mechanism for Ca^{2+} dependent regulation of Pax3 transcriptional activity.

$InsP_3R$ Ca^{2+} release channel

The inositol 1,4,5-triphosphate ($InsP_3$) receptor Ca^{2+} release channel is a ubiquitous signalling system regulating calcium mobilisation from the ER (Berridge et al., 2000). CIB1 has been identified as a ubiquitously expressed ligand for all mammalian isoforms of $InsP_3R$, interacting in a Ca^{2+}-sensitive manner (White et al., 2006). In the absence of $InsP_3$, CIB1 activated channel gating. Pre-exposure of the receptor to CIB1, however, reduced the number of channels available for subsequent activation by $InsP_3$. In addition, overexpression of CIB1 reduced the amplitude of ATP-induced calcium fluxes in cells (White et al., 2006). Hence, CIB1 appears to be both an activator and inhibitor of Ca^{2+} release from $InsP_3R$.

Myo1c

Myo1c is a member of the myosin-I family of proteins. These members commonly bind phosphoinositides and link the actin cytoskeleton to cellular membranes (Tang et al., 2007). CIB1 and myo1c were found to extensively co-localise in cells, and through pull-down analysis, CIB1 was found to interact with myo1c in its IQ motifs, and here it competes for

binding with CaM in the presence of calcium (Tang et al., 2007). The IQ motifs in myo1c are thought to target this protein to specific subcellar locations through interactions with binding partners (Cyr et al., 2002). Hence, these authors hypothesise CIB1 to play a role in specifying a subcellular localisation of myo1c.

Caspase-2S

Caspase-2S and caspase-2L are derived from alternative splicing of the mRNA encoding caspase-2. These two proteins appear to have antagonising roles, with caspase-2L inducing apoptosis, while caspase-2S antagonises cell death (Wang et al., 1994). A yeast two-hybrid system identified CIB1 as an interaction partner for Caspase-2S, with CIB1 able to partially suppress the activation of Caspase-2L *in vitro* (Ito et al., 2000). Hence, it was suggested that CIB1 may be involved in caspase-2S mediated cellular survival, however the inhibitory mechanism for its suppression of Caspase-2L remained unclear.

In addition to these above mentioned interactions, CIB1 has been identified as binding to a number of other cellular proteins, although many of these remain uncharacterised. CIB1 was demonstrated to interact with G1P3 an interferon inducible gene localized to the mitochondria in human senescent fibroblasts (Tahara et al., 2005). Although the function of this protein remains largely unknown, G1P3 is expressed at high levels in immortalized cells, appearing to have an anti-apoptotic effect (Tahara et al., 1994). Further characterisation of a potential role for CIB1 in the G1P3 induced suppression of apoptosis has not yet been reported. CIB1 has also been identified as interacting with the C-terminal domain of the two polo family of cell cycle associated kinases, Fnk and Snk (Kauselmann et al., 1999). These two proteins are typically believed to function in the cell cycle (Glover et al., 1998) but were also proposed to be involved in altering the molecular composition and structure of neurons in order to stabilize changes in synaptic strength (Kauselmann et al., 1999). However, a role for CIB1 stabilisation of synaptic plasticity is yet to be elucidated. In addition CIB1 was identified through a yeast two-hybrid study as interacting with an uncharacterised protein NBR1 (Whitehouse et al., 2002). This protein is such named due to its location, 'next to BRCA1', yet its function remains largely unknown. Lastly, CIB1 was also identified as a binding partner for DNA-dependent protein kinase (DNA-PK_{CS}), a serine/threonine kinase, again through a yeast two-hybrid approach (Wu and Lieber, 1997).

However, confirmation of this interaction in cells was not examined, nor was a function of this putative interaction.

As these above-mentioned interactions show, CIB1 has been identified as a binding partner for a number of cellular proteins, with apparent diverse roles and cellular expressions. Hence, a wide regulatory role for CIB1 in cellular signalling events is likely.

1.11.5 CIB1 Knock-out Mice

To attempt to gain a better understanding of the function of CIB1 in whole animals, Yuan et al. (2006a) generated CIB1$^{-/-}$ mice. These mice grew normally and were macroscopically indistinguishable from their *wild-type* littermates. However, one observable defect in these mice as the inability of male CIB1$^{-/-}$ mice to reproduce due to a disruption in the haploid phase of spermatogenesis. These mice also showed reduced numbers of germ cells in seminiferous tubes and no detectable sperm. In addition, embryonic fibroblasts isolated from these mice (MEFs) proliferated more slowly than those generated from *wild-type* mice, and an increase in germ cell apoptosis was detected. However, this potential anti-apoptotic function of CIB1 is still unclear as these results are in disagreement with an earlier study showing an increase in cell death upon expression of CIB1 in HeLa cells (Stabler et al., 1999). Further studies utilising endothelial cells isolated from these mice have also suggested a role for CIB1 in angiogenesis, with CIB1 depleted endothelial cells undergoing less proliferation, migration and tubule formation in comparison to *wild-type* endothelial cells (Zayed et al., 2007). These effects were seen in association with decreased PAK1 activation, and also attenuation of ERK1/2 phosphorylation in the CIB1 depleted cells.

1.11.6 CIB1 may Function as a Calcium-Myristoyl Switch Protein

One particularly notable finding that may provide insight to the function of CIB1 *in vivo* was the discovery that CIB1 is myristoylated at its N-terminal glycine residue in HeLa cells (Stabler et al., 1999). This lead to speculation that this protein may function as a calcium-myristoyl switch. Although this class of proteins is poorly characterized, it is generally understood that the in absence of bound Ca^{2+}, the myristoyl group is sequestered into a

hydrophobic pocket in the protein. However, upon binding Ca^{2+}, the protein undergoes a conformation change conferring a dual effect; firstly in enabling the interaction of the protein with target substrates, and secondly to cause the extrusion of the myristoyl group from its original sheltered groove, targeting the protein and any newly bound interacting partner to intracellular membranes (Figure 1.9) (Meyer and York, 1999).

Evidence for the membrane association of CIB1 comes from cell fractionation studies, whereby CIB1 fractionated exclusively with membrane fractions in both nucleated and non-nucleated cells (Stabler et al., 1999). However, in one study that attempted to determine the calcium-myristoyl switch function of CIB1, this protein failed to translocate to intracellular membranes in response to Ca^{2+} mobilisation induced by ionomycin (Blazejczyk et al., 2006). However, the results from this study may be subject to scrutiny for several reasons. Firstly is the use of GFP-tagged CIB1. Although the GFP was fused to the C-terminus of CIB1 and therefore should not alter its myristoylation, the presence of this large fusion partner may affect the localisation of this protein, and impede its normal movement within the cell (Hanson and Ziegler, 2004). Also, it should be noted that in these studies, no obvious translocation was observed in response to ionomycin for VILIP-1, a known calcium-myristoyl switch family member (Blazejczyk et al., 2006), shedding further doubts on the validity of these findings. Thus, the potential role of CIB1 as a Ca^{2+}-myristoyl switch protein remains undetermined.

1.11.7 Does CIB1 Function to Translocate SK1 to the Plasma Membrane?

The possible function of CIB1 as a calcium-myristoyl switch protein in accordance with its similarities shared with CaM, allows for speculation that CIB1 may have a role in the phosphorylation-dependent translocation of SK1 to the plasma membrane. A possible model is one where enhanced intracellular Ca^{2+}, resulting either from SK1 activation or other SK1-independent mechanisms, cause CIB1 to undergo a conformational change. This conformational change may functions to both enable its association with SK1, but also result in extrusion of its myristoyl group, targeting this newly formed complex to the plasma membrane. Once at the plasma membrane, phosphorylated SK1 may be retained at this location through association with PA or PS in the lipid bilayer. This membrane localisation of SK1 likely facilitates S1P production, by bringing activated SK1 into close proximity

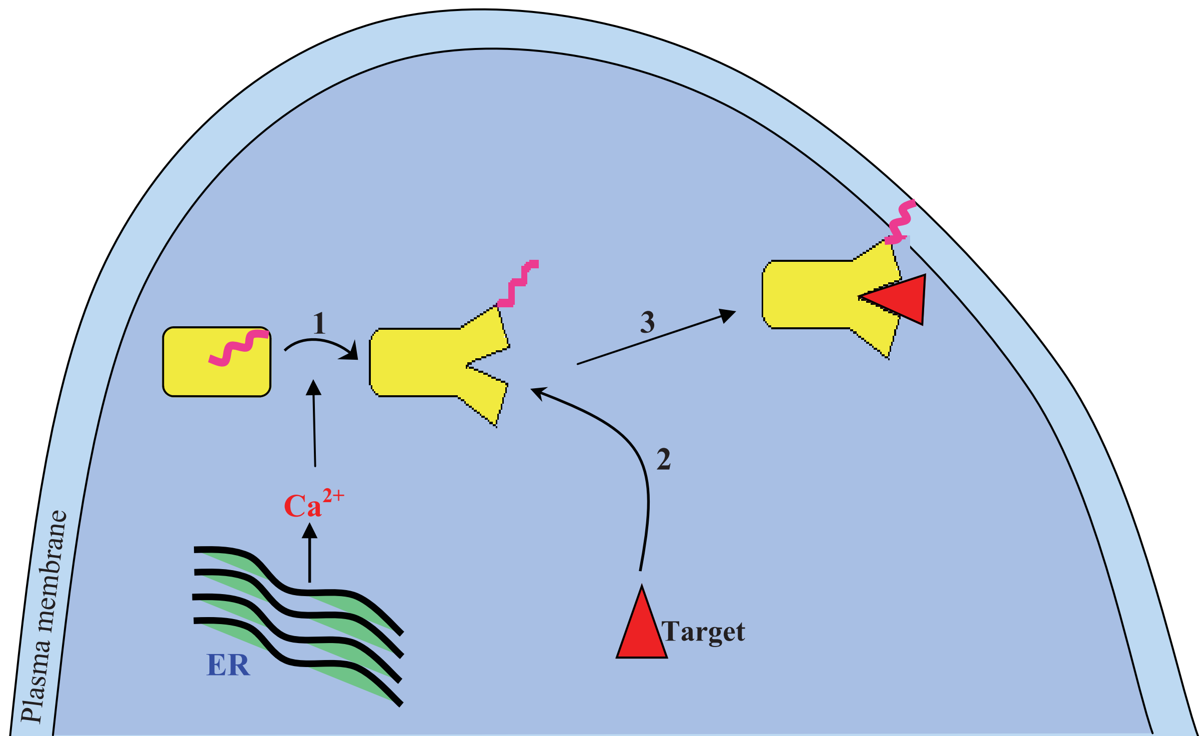

Figure 1.9. The proposed Ca^{2+}-myristoyl switch mechanism.

In the absence of calcium, these proteins are hypothesized to sequester their myristoyl motif (pink) into a hydrophobic pocket. Upon mobilization of calcium, the association of calcium with these proteins results in a conformational change, enabling interaction with target substrates, and resulting in extrusion of the myristoyl motif, targeting both the Ca^{2+}-myristoyl switch and target protein to intracellular membranes.

with sphingosine stores. Hence, through a role in SK1 plasma membrane targeting, CIB1 may also mediate the agonist-induced production of S1P, and regulate downstream events associated with this lipid second messenger. In considering this model, however, one must also note the existence of three CIB1 homologs in the human genome.

1.11.8 CIB1 Homologs

Gentry et al. (2005) noted that several proteins in the sequence database from arthropods to humans share significant sequence homology to CIB1. In humans, these proteins are termed CIB2, CIB3 and CIB4, and share 59%, 62% and 64% amino acid sequence similarity with CIB1 respectively (Figure 1.10). There is currently, however, very little published data on CIB2 and CIB3 and no published data on CIB4.

From sequence analysis, all CIB homologs appear to contain the conserved residues for Ca^{2+} binding in EF-hands -3 and -4 (Gentry et al., 2005). Similarly, all CIB isoforms have a large insertion (eight amino acids) in EF1 within the loop that normally binds Ca^{2+}, strongly suggesting that this EF-hand cannot bind Ca^{2+} in any of these proteins. In addition, all CIB proteins appear to contain an equivalent residue to the Pro62 of CIB1 which is important for the overall structure of the hydrophobic binding pocket (Gentry et al., 2005). However, somewhat surprisingly, the EF2 loop (between helices H4 and H5) in CIB2 and CIB3 contains more acidic residues than that of CIB1, suggesting the possibility that these two homologs may bind an additional Ca^{2+} ion within this region (Gentry et al., 2005). Similar to CIB1, CIB2 and CIB3 contain an N-terminal myristoylation consensus (MGXXXS/T) (Gentry et al., 2005), suggesting that these isoforms are likely to be modified with this fatty-acid moiety. CIB4, however, deviates from this consensus with an arginine at amino acid position six. Hence, it is not predicted to be a target for this modification (Utsumi et al., 2004). Further sequence analysis of CIB1 and its homologs with a number of other mammalian Ca^{2+} binding proteins revealed that these proteins are much more similar to one another than to any of their other related proteins (Gentry et al., 2005). Consequently, this led this group to conclude that the CIB-like proteins form their own family of EF-hand containing proteins, distinct from both the calcineurin B-like and NCS proteins.

NOTE:
This figure is included on page 29a of the print copy of the thesis held in the University of Adelaide Library.

Slightly modified from Yamniuk and Vogel, 2006

Figure 1.10. Multiple sequence alignment of the CIB isoforms with CnB.
Each of the residues incorporated into the EF hands are shown above the sequences, with the alpha-helical regions for Ca^{2+} bound CIB1 also shown. Conserved residues between the isoforms are highlighted and the Ca^{2+} coordinating residues are coloured in red. Myristoylation consensus sequences are highlighted in purple.

The CIB2 gene is localised to the q24 region of chromosome 15 (Seki et al., 1999). Through RT-PCR analysis, CIB2, like CIB1, appears to be widely expressed in a number of human tissues (Seki et al., 1999). Only recently, however, has CIB2 reappeared in the literature, with a study identifying CIB2 mRNA as being down regulated in a mouse muscular dystrophy model (Hager et al., 2008). Congenital muscular dystrophy type 1A is caused by genetic mutation in laminin α2, with this protein able to partner with the integrin α7Bβ1. CIB2 was found to interact with the integrin α7Bβ1D and colocalise with the integrin α7B subunit at the sarcolemma and at the neuromuscular and myotendinous junctions (Hager et al., 2008). Additionally, CIB2 was confirmed to bind Ca^{2+}, as anticipated from its EF-hand structure. These authors hypothesised a role for CIB2 as an effector of integrin α7Bβ1D signalling in skeletal muscle. Notably, this is interesting as it parallels the interaction of CIB1 with the platelet-specific integrin αIIbβ3, regulating outside-in signalling in platelet aggregation (Yamniuk and Vogel, 2006). Hence, this leads to the question of whether CIB1 and CIB2 may have similar functions in various tissues/ cells.

Although no studies have examined the expression or function of CIB3 to date, CIB3 mRNA was found to be upregulated in CIB1 deficient platelets (Denofrio et al., 2008). Furthermore, this study showed that recombinant CIB1, -2 and -3 bound specifically to a αIIb cytoplasmic tail peptide *in vitro*. As no defect in platelet aggregation was observed in these CIB1 knock-out mice, these authors suggested that compensation between CIB1 and CIB3 can occur, resulting in normal αIIbβ3 signalling and platelet function.

Hence, from the very limited studies on the CIB family members, it appears that these proteins most likely share a very similar structure with CIB1, and may function similarly in cells.

1.12 Hypothesis

The translocation of SK1 from the cytoplasm to the plasma membrane following its agonist-induced activation is integral for a number of its biological signalling outcomes, including those leading to cellular proliferation, anti-apoptosis and neoplastic transformation.

I hypothesise that this rapid, agonist-induced translocation of SK1 to the plasma membrane is mediated by one or more of the CIB isoforms. In addition, through facilitating this translocation event, one or more of the CIB proteins will contribute to biological events associated with this pathway, such as SK1-mediated anti-apoptosis and oncogenesis.

1.13 Aims

1. Characterise each of the CIB isoforms in terms of their interactions with SK1

2. Determine the role of the CIB isoforms in the translocation of SK1 to the plasma membrane upon agonist stimulation.

3. Determine if any/ all of the CIB isoforms contribute to biological responses of SK1 translocation such as anti-apoptosis and oncogenesis.

Chapter 2: General Materials and Methods

2.1 Cell Culture

Human embryonic kidney cells (HEK293T) were cultured in Dulbecco's modified Eagles medium (Gibco) containing 10% bovine calf serum (JRH Biosciences, Lenexa, KS), penicillin (1.2 mg/ml) and streptomycin (1.6 mg/ml).

HEK293T cells were transiently transfected using the calcium phosphate precipitation method (Graham and van der Eb, 1973), harvested and lysed 24 hours later by sonication (3 watts for 30s at $4^{o}C$) in 50 mM Tris/HCl pH 7.4 containing 150 mM NaCl, 2 mM Na_3VO_4, 10 mM NaF, 1 mM EDTA, 10% glycerol, 0.05% Triton X-100, 10 mM β-glycerophosphate, 1 mM DTT and protease inhibitors (Complete; Roche Molecular Biochemicals) (Extraction Buffer). Protein concentrations in cell homogenates were determined with Bio-Rad Protein Assay (Bio-Rad Laboratories) using bovine serum albumin as standard.

Human cervical carcinoma (HeLa) cells, DU145 and MCF-7 breast carcinoma cells or NIH3T3 fibroblasts were cultured and harvested in the same manner, and transfected with Lipofectamine 2000 (Invitrogen) using the manufacturer's protocol.

2.2 Generation of Pooled Stable Cell Lines

NIH3T3 fibroblasts or HeLa cells were transfected with the appropriate DNA construct as described above. A mock transfected plate was included, where these cells were exposed to the transfection reagent, but no DNA. One day post transfection, cells were passaged 1:2 and 0.8 mg/ml G418 (Gibco) selection was applied. Media and selection was replaced every 3 days, with the cells passaged when appropriate. Mock cells died between 3-7 days following addition of selection. Cells were selected for at least 3 weeks prior to use in further experiments, with protein expression first confirmed by Western blotting.

2.3 Generation of Expression Constructs

2.3.1 CIB1 Expression Constructs

A yeast two-hybrid screen, using a human leukocyte cDNA library and full-length human SK1 as bait was previously performed (Zebol et al., 2009) and isolated a full length CIB1 cDNA.

Human CIB1 (Genbank™ Accession number U82226) was hemaglutanin (HA) epitope-tagged at the 3' end by *Pfu* DNA polymerase chain reaction using cDNA isolated from the yeast two-hybrid screen with oligonucleotide primers: 5'-CCCAAGCTTGCCACCATGGG-GGGCTCGGGCAG-3' and 5'-GGGGATCCTCAAGCGTAATCTGGAACATCGTATG-GGTACAGGACA ATCTTAAAGGAGC-3'. The resultant product was cloned into pcDNA3 (Invitrogen) following digestion with HindIII and BamHI.

The myristoylation mutant $CIB1^{G2A}$ was generated from CIB1(HA) in pcDNA3 by QuikChange mutagenesis using the primers 5'-AGCTTGCCACCATGGCGGGATCCGGC-AGTCGCCTGTCC-3' and 5'-GGACAGGCGACTGCCGGATCCCGCCATGGTGGCAA-GCT-3'.

A second non-myristoylated CIB1 (HA-tagged at the *N*-terminus and termed HA-CIB1) was generated by PCR using: 5'-GAAGATCTTCATGGGGGGCTCGGG CAG-3' and 5'-GGGGTACCCCTCACAGGACAATCTTAAAGGA-3' oligonucleotide primers. The PCR product was cloned into pCMV(HA) (BD Biosciences Clontech) following digestion with BglII and KpnI.

To generate the recombinant GST-CIB1 fusion protein, CIB1 cDNA was PCR amplified with the following oligonucleotide primers 5'-CCCGGATCCGCCACCATGGGGGGCTC-GGGCAG-3' and 5'-GGGCTCGAGTCACAGGACAATCTTAAAGGA-3', and the resultant product cloned into pGEX4T2 (GE Healthcare) following digestion with BamHI and XhoI. Sequencing verified the integrity of all cDNAs.

2.3.2 CIB2 Expression Constructs

Human CIB2 (Genbank Accession number NM_006383) was amplified from placenta cDNA and HA epitope-tagged at the 3' end by *Pfu* DNA polymerase chain reaction with oligonucleotide primers 5'-TAGGATCCGCCACCATGGGGAACAAGC-3' and 5'-TAGGATCCTCAAGCGTAATCTGGAACATCGTATGGGTAGATCCGGATG TGGAAAGTG-3'. The PCR product was cloned into pcDNA3 following digestion with BamHI. Restriction digestion and sequencing verified the integrity and orientation of the cDNA.

A recombinant GST-CIB2(HA) fusion protein was generated by sub-cloning into pGEX4T2 following digestion with BamHI. Restriction digestion verified the orientation of the cDNA.

2.3.3 CIB3 Expression Constructs

Human CIB3 (Genbank Accession number NM_054113), cDNA was purchased as pCR4-CIB3Blunt-TOPO (Geneart). This CIB3 construct was HA epitope-tagged at the 3' end by *HiFi* PCR with oligonucleotide primers T3 and 5'-TAGGATCCTCAAGCGTAATCTGGA-ACATCGTATGGGTAGATCCGGATGTGGAAAGTG-3'. The PCR product was cloned into pcDNA3 following digestion with KpnI and BamHI. Sequencing verified the integrity of the cDNA.

A recombinant GST-CIB3(HA) fusion protein was generated by PCR with the following oligonucleotide primers 5'-TAGAATTCATGGGCAACAAGCAGACAGTCTTCACACA-CGAGCAGCTGGAAGC-3' and SP6 using pcDNA3-CIB3(HA) as template. The PCR product was cloned into pGEX4T1 following digestion with EcoRI. Restriction digestion and sequencing verified the integrity and orientation of the cDNA.

2.3.4 CIB4 Expression Constructs

Human CIB4 (Genbank Accession number NM_001029881) was amplified from placenta cDNA and HA epitope-tagged at the 3' end by *Pfu* PCR with oligonucleotide primers 5'-TAGGATCCGCCACCATGGGGCAATGCTTGAGG-3' and 5'-TAGGATCCTCAAG-CGTAATCTGGAACATCGTATGGGTAGCATCCCCAGAAGTGAATC-3'. The PCR product was cloned into pcDNA3 following digestion with BamHI. Restriction digestion and sequencing verified the integrity and orientation of the cDNA.

A recombinant GST-CIB4(HA) fusion protein, was generated by sub-cloning into pGEX4T2 following digestion with BamHI. Restriction digestion verified the orientation of the cDNA.

Mammalian expression constructs encoding human SK1, $SK1^{S225A}$, $SK1^{F197A/L198Q}$ and EGFP-SK1 (all tagged with a *C*-terminal FLAG epitope) were previously generated (Pitson et al., 2000a; Pitson et al., 2003; Sutherland et al., 2006). pGS5-V12HRas was generated as previously described (Marte et al., 1997).

2.4 Generation of Recombinant GST-CIB1, GST-CIB2, GST-CIB3 and GST-CIB4

E. coli JM109 transformed with pGEX4T2-CIB1/-2/-3/-4 plasmid were grown overnight in Luria broth containing 100 mg/L ampicillin at 37°C with shaking. The cultures were then diluted 1 in 10 into the same medium and grown at 37°C for 1 h with shaking until reaching an A_{600} of ~0.6. Expression of the GST-CIB protein was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 1 mM, and the culture incubated for an additional 1 h. The cells were harvested by centrifugation at 6000 × *g* for 15 min at 4°C and lysed by sonication (three 30 s pulses of 5 watts) in extraction buffer containing 1% Triton X-100. The lysate was clarified by centrifugation at 20,000 × *g* for 30 min at 4°C to remove cell debris. Glutathione (GSH)-Sepharose (Amersham) was added, and the mixture was incubated at 4°C for 1 h with constant agitation. The glutathione-Sepharose beads were washed three times with cold phosphate-buffered saline (PBS), and

GST-CIB protein remaining quantitated with Coomassie Brilliant Blue staining following SDS-PAGE using BSA as standard.

2.5 Pull-down Analyses

Pull-down analyses were performed by incubating 1 μg recombinant SK1 (Pitson et al., 2002) with 1 μg of the purified GST-CIB protein or GST alone bound to GSH-Sepharose in the presence of 2 mM $CaCl_2$, 2 mM $MgCl_2$ or 2 mM EGTA for 1 h at 4°C with constant agitation. Inhibition of the recombinant SK1-CIB1 interaction was achieved by addition of 100 μM N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7; Calbiochem) to the mix. Alternatively, lysates from untransfected DU145 cells, or from HEK293T cells transiently transfected with the protein of interest were diluted into 33 mM Tris/HCl (pH 7.4) buffer containing 100 mM NaCl, 10% glycerol and 0.033% Triton X-100 and incubated with the purified GST proteins under the same conditions. The GSH-Sepharose beads were pelleted by centrifugation at 3000 × g and washed three times in the same buffer and bound protein then resolved by SDS-PAGE and visualized by Western blotting. SK1, $SK1^{S225A}$ and $SK1^{F197A/L198Q}$ were all detected via the FLAG epitope while recombinant SK1 was detected via its 6xHIS epitope.

2.6 Western Blotting

The lysate of interest was heated at 100ºC for 5 mins with a final concentration of 1 x sample buffer containing 1 mM Tris/HCl (pH 8.0), 1 mM EDTA, 20 mM DTT, 1% SDS (w/v), 10% glycerol and 0.02% bromophenol blue. Alternatively, 20 µl of 5 x sample buffer was added to GSH-Sepharose beads from pull-down analyses and heated at 100ºC for 5 mins. In order to achieve maximal protein loading for phospho-ERK blots, cell pellets were lysed directly in 80-100 µl 1x sample buffer, boiled for 10 mins and debris removed by centrifugation. Samples were loaded into wells of a 12% SDS-polyacrylamide gel and run at 200V until the dye front had run off the bottom of the gel. Proteins were transferred to nitrocellulose membrane through a wet transfer method, at 250 mA for 1 h. Membranes were blocked with PBS containing 5% Skim milk powder and 0.1% Triton X-100 for 15

mins at room temperature, or overnight at 4ºC prior to exposure to the appropriate primary and secondary antibodies.

2.7 Antibodies

M2 anti-FLAG and anti-HA antibodies were from Sigma-Aldrich, anti-IκBα and anti-His antibodies were from Santa Cruz, anti-phospho-ERK antibodies were from Cell Signalling, anti-α-tubulin antibodies were from Abcam, and HRP-conjugated anti-mouse and anti-rabbit IgG were from Pierce Chemical Co. Anti-SK1 and phospho-SK1 antibodies were generated as described previously (Pitson et al., 2003). Alternate anti-SK1 antibodies, used to immunoprecipitate endogenous SK1, were purchased from Santa Cruz. Anti-CIB1 polyclonal antibodies were raised in rabbits against GST-CIB1 protein produced in *E. coli* (described above) using methods previously described (Pitson et al., 2003). Briefly, rabbits were inoculated with four doses of 0.5 mg of purified GST-CIB1 protein at 3 week intervals, and serum collected 10 days following the final inoculation.

2.8 Affinity Purification of CIB1 Antibodies

E. coli cell lysate containing approximately 0.5 mg GST-CIB1 was incubated with 1 ml of GSH-Sepharose for 30 min at 4°C with constant mixing. The GSH-Sepharose and associated GST-CIB1 were then crosslinked with dimethyl pimelimidate (Pierce) as previously described (Bar-Peled and Raikhel, 1996). Rabbit anti-CIB1 serum was then applied to the resin and mixed at 4°C for 1 h. The resin was then washed with 10 mM Tris/HCl buffer (pH 7.5) followed by 10 mM Tris/HCl buffer (pH 7.5) containing 0.5 M NaCl. The CIB1 antibody was then eluted from the resin with 100 mM glycine buffer (pH 2.5), with immediate neutralisation with 1 M Tris/HCl buffer (pH 8.8). Concentration of the antibody was then achieved by precipitation through the addition of ammonium sulphate to 50% saturation at pH 7.4 and incubating the mix for 2 days at 4°C. The precipitated antibody was pelleted by centrifugation at 20,000 × *g* for 45 min at 4°C, and the pellet resuspended in 1 ml of 100% saturated ammonium sulphate, pH 7.4.

2.9 Immunoprecipitation

Lysates from HEK293T cells expressing either FLAG-tagged SK1 alone or in combination with the appropriate HA-tagged CIB protein were prepared in 33 mM Tris/HCl (pH 7.4) buffer containing 100 mM NaCl, 10% glycerol, 0.033% Triton X-100 and 2 mM $CaCl_2$ and incubated with anti-HA antibodies for 1 h at 4°C with constant agitation. The immune complexes were then captured by incubation with protein A-Sepharose (GE Healthcare) for a further 1 h at 4°C with constant agitation. Protein A-Sepharose beads were washed 3 times in the same buffer then subjected to SDS-PAGE and associated SK1 visualized by Western blotting via the FLAG epitope. To co-immunoprecipitate the endogenous SK1-CIB1 complex, lysates from MCF-7 cells in 33 mM Tris/HCl (pH 7.4) buffer containing 100 mM NaCl, 10% glycerol, 0.033% Triton X-100 and 2 mM $CaCl_2$ were incubated with anti-SK1 antibodies (Santa Cruz, CA) and protein A MicroBeads (Miltenyi Biotec) for 1 h on ice. As a negative control, a lysate sample containing no antibodies was simultaneously incubated with protein A MicroBeads. The immune complexes were captured using a 20 µ MACS® Separation Column (Miltenyi Biotec) and washed according to the manufacturers instructions. Elution of the immune complexes was achieved through application of hot SDS-PAGE loading buffer to the column. Samples were subjected to SDS-PAGE and the immunoprecipitated SK1 and associated CIB1 visualized by Western blotting using anti-SK1 and anti-CIB1 antibodies respectively.

2.10 Detection of CIB Myristoylation

One day after transfection HeLa cells were incubated in DMEM containing 5% dialysed fetal bovine serum for 2 h. Sodium pyruvate to a final concentration of 5 mM and 0.25 mCi ^{3}H-myristic acid (PerkinElmer) were then added to the media and the cells incubated at 37°C, 5% CO_2 for a further 5 h. The cells were harvested by scraping into cold PBS and then lysed in Extraction Buffer (see section 2.1) containing 1% Nonidet P-40 Substitute (Sigma-Aldrich) by 5-passages through a 26 gauge needle. Lysates were incubated on ice for 30 min and then cell debris cleared by centrifugation at 13,000 × g for 20 min at 4°C. CIB proteins were immunoprecipitated via their HA tag with anti-HA antibodies and a mix of protein A-and protein G-Sepharose as described above. The sepharose beads were

washed three times in Extraction Buffer containing 1 % Nonidet P-40 substitute, then subjected to SDS-PAGE. The gel was fixed for 30 min in 10% (v/v) acetic acid with 40% (v/v) ethanol in water, followed by incubation in Amplify solution (GE Healthcare) for 1 h with agitation. The gel was then dried for 90 min at 65°C under vacuum, and exposed to film for 6 weeks at -80°C.

2.11 Immunofluorescence

One day after transfection, HeLa cells were plated into poly-L-lysine (Sigma) coated 8-well glass chamber slides at 10^4 cells / well and incubated for 24 h. Cells were then stimulated with 1 μg/ml phorbol 12-myristate 13-acetate (PMA; Sigma) for 30 min, 2 μM ionomycin (Calbiochem) for 2 min or 5 μM thapsigargin (Calbiochem) for 5 min. Ca^{2+} chelation was achieved by cell treatment with 50 μM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N′*,*N′*-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA/AM) (Calbiochem) for 30 min prior to stimulation with PMA. For inhibition of SK1 translocation, cells were treated with 100 μM W7 for 5 min prior to PMA stimulation. Following stimulation, cells were fixed for 10 min with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton-X-100 in PBS for 10 min, and then incubated with either anti-HA or anti-SK1 antibodies in PBS containing 3% BSA and 0.1% Triton-X-100 for 1 h. The immunocomplexes were then detected with Alexafluor-594 conjugated anti-mouse or anti-rabbit IgG. Localisation of overexpressed SK1 was visualized via its EGFP fusion. Slides were mounted with Dako Fluorescent Mounting Medium. Fluorescence microscopy was performed on an Olympus BX-51 microscope equipped with fluorescein excitation filters (494 nm and 550 nm) acquired to a Cool Snap FX charge-coupled device camera (Photometrics). All images were acquired at room temperature at 40x magnification using V++ acquisition software (Digital Optics). For quantitation of membrane localisation, raw gray values from uniformly sized areas were measured from eight random membrane locations per cell using Cell∧R analysis software (Olympus). Exposure times and total cell fluorescence were consistent between images used for quantitation. Background fluorescence was measured from an area on the slide where no cells were seeded. Mean membrane fluorescence was calculated after subtraction of background fluorescence.

2.12 siRNA Knock-Down of CIB1

CIB1 siRNA duplexes were purchased from Invitrogen with the following sequences: siRNA1: 5'-UAAUGGGACUUGAUGUCUGGCGUGG-3' and siRNA2: 5'AUGACGUGCUGGAACUCAGAGAGGU-3'. HeLa cells were transfected with either CIB1 siRNA or Validated Stealth^{TM} Negative Control (Invitrogen) using HiPerFect Transfection Reagent (Qiagen) according to the manufacture's protocol, and incubated for 48 h prior to harvesting and use in further experiments. All results using siRNA knock-down of CIB1 were validated by repetition with CIB1 siRNA2.

2.13 Quantitation of S1P

S1P levels were measured using fluorescence derivatisation followed by high-performance liquid chromatography (HPLC), essentially as described previously (Ruwisch et al., 2001). Briefly, S1P was extracted using a two-step Bligh-Dyer extraction, initially under alkaline conditions where S1P largely partitions to the aqueous phase, and then under acidic conditions, where S1P partially partitions to the organic phase. C17-S1P (Avanti Polar Lipids, Alabaster, AL) was used as an internal standard. The organic phase was then evaporated and the dried S1P dissolved in 275 µl methanol/70 mM K_2HPO_4 (3:1). Fluorescent derivatisation of S1P was achieved by the addition of 25 µl of a derivatisation mixture containing 5 mg *o*-phthalaldehyde, 100 µl ethanol, 5 µl ß-mercaptoethanol in 5 ml of 3% boric acid (pH 10.5). The mixture was then incubated at room temperature for 15 min prior to HPLC analysis. Samples (100 µl) were applied to an X-bridge C18 column (Waters, Milford, MA) equilibrated with methanol/70 mM K_2HPO_4 (3:1) using a flow rate of 1.5 ml/min. S1P was then eluted from the column with a 10 min linear gradient to methanol, with fluorescence measured at emission and excitation wavelengths of 455 nm and 340 nm, respectively. The resulting profiles were evaluated using the Millenium[32] Chromatography Manager software (Waters).

2.14 TNF-α-Induced Apoptosis

HeLa cells were treated with 2 ng/ml TNF-α and 1 μg/ml cycloheximide (Sigma) for 18 h. As HeLa cells lose their adherence during programmed cell death (Stabler et al., 1999), both floating (apoptotic) and live attached cells were then counted as a measure of apoptosis. To correlate floating cells with apoptotic cells, Annexin V staining was performed on non-permeabilized cells, with 99% of floating cells showing positive cell surface staining for this apoptosis marker. As a subsequent measure of apoptosis, caspase-3/7 activity was measured from cell lysates prepared from the above mentioned cells using a Caspase-Glo 3/7 assay (Promega) according to the manufacturer's protocol. The cell counts were used to standardise between samples, to give a measure of caspase-3/7 activity per cell.

2.15 NFκB Reporter Assay

HeLa cells were transfected with either Stealth negative control or CIB1 siRNA in combination with either pIgKluc for NF-κB-dependent expression of firefly luciferase or control pTK81 vector lacking the NF-κB binding sites (Himes et al., 1996). pRL-TK (Renilla luciferase; Promega) was included in each transfection to standardise transfection efficiency. Two days following transfection, cells were stimulated with 0.5 ng/ml TNF-α for 4 h and a dual-luciferase reporter assay (Promega) carried out according to the manufactures protocol. Firefly luminescence was standardised to Renilla and calculated relative to cells expressing pTK81.

2.16 Focus Formation

For generation of pooled stables, NIH3T3 cells were transfected with CIB1, CIB1^{G2A} or empty vector and selected with 0.8 mg/ml neomycin (G418) for 21 days prior to use, with media and selection changed every 2-3 days. For focus formation assays, selection was removed and cells were seeded into 6-well dishes. Once confluence was achieved, cells were cultured for 3-4 weeks, with media replaced every 2-3 days in the presence or absence of 5 μM SK-I (Calbiochem). For Ras-induced transformation, one day after seeding, the

pooled stable cell lines described above were transfected with V12-Ras and cultured as above. Foci were scored after fixing with methanol and staining with 0.05% methyl violet.

Chapter 3: Translocation of sphingosine kinase 1 to the plasma membrane is mediated by calcium and integrin binding protein 1

3.1 Abstract

Sphingosine kinase 1 (SK1) plays an important role in many aspects of cellular regulation. Most notably, elevated cellular SK1 activity leads to increased cell proliferation, protection from apoptosis, and induction of neoplastic transformation. Translocation of SK1 from the cytoplasm to the plasma membrane has been previously shown to be integral for oncogenesis mediated by this enzyme. The molecular mechanism mediating this translocation of SK1 has, however, remained undefined. Here, I demonstrate a direct role for calcium and integrin binding protein 1 (CIB1) in this process. I show that CIB1 interacts with SK1 in a Ca^{2+} dependent manner at the previously identified 'calmodulin binding site' of SK1. I also demonstrate that CIB1 functions as a Ca^{2+}-myristoyl switch, providing a mechanism whereby it translocates SK1 to the plasma membrane. Both siRNA knock-down of CIB1 and the use of a dominant-negative CIB1 I generated prevented the agonist-dependent translocation of SK1. Furthermore, I demonstrate the requirement of CIB1-mediated translocation of SK1 in controlling cellular sphingosine-1-phosphate generation and associated anti-apoptotic signalling. Finally, I show the ability of CIB1 to induce neoplastic transformation of cells in an SK-dependent manner. Furthermore, the well documented oncogenic properties of V12-H-Ras, previously shown to be partially SK1 dependent, were also inhibited by dominant-negative CIB1. These findings point to CIB1 as a potential new target in cancer therapies.

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See Appendix 1 for reprint and author contributions.
Additional data not included in this publication are shown in Figures 3.15 and 3.16.

3.2 Introduction

Sphingosine kinase 1 (SK1) catalyses the formation of sphingosine-1-phosphate (S1P), a bioactive phospholipid that mediates a wide variety of cellular processes. Elevated cellular S1P has been shown to be pro-proliferative and anti-apoptotic (Olivera et al., 1999), and considerable evidence now exists implicating SK1 in cancer. In particular, overexpression of SK1 in NIH3T3 fibroblasts leads to a transformed phenotype and the ability to form tumours in mice, with SK1 activity also involved in oncogenic H-Ras mediated transformation (Xia et al., 2000). Furthermore, suppression of cellular SK1 activity by genetic or pharmacologic approaches has been shown to significantly reduce tumour growth *in vivo* in mice (French et al., 2003; Kohno et al., 2006; Kawamori et al., 2009) and also sensitise tumour cells to other chemotherapeutics (Pchejetski et al., 2005).

While SK1 has intrinsic catalytic activity (Pitson et al., 2000c), its further activation is required for oncogenic signalling (Pitson et al., 2005). This activation, brought about through phosphorylation at Ser225 by ERK1/2, not only increases the catalytic activity of SK1, but also results in its translocation from the cytoplasm to the plasma membrane (Pitson et al., 2003) which is essential for the oncogenic signalling by this enzyme (Pitson et al., 2005; Wattenberg et al., 2006).

Although critical in understanding SK1 induced oncogenesis, the mechanisms regulating agonist-induced translocation of SK1 to the plasma membrane are poorly understood. Studies have suggested that SK1 associates with phosphatidylserine in a phosphorylation-dependent manner (Stahelin et al., 2005). While this may facilitate retention of SK1 at the plasma membrane, its role in mediating the initial rapid agonist-induced translocation of SK1 has not yet been established. Several studies have identified a Ca^{2+} dependent interaction between Calmodulin (CaM) and SK1 *in vitro* (Pitson et al., 2000b; Olivera et al., 1998; Pitson et al., 2001; Pitson et al., 2002). This protein has since been indirectly implicated in this process since W7, a CaM inhibitor, blocked SK1 translocation (Young et al., 2003), as did mutation of the CaM-binding site of SK1 (Sutherland et al., 2006). Evidence for a direct role for CaM in SK1 translocation, however, has not been described. Furthermore, CaM predominately moves from the cytoplasm to the nucleus, not the plasma membrane, in response to cellular Ca^{2+} fluxes (Chin and Means, 2000; Thorogate and Torok, 2007) raising doubts over the role of CaM in SK1 localisation. The actin

crosslinking protein Filamin A (FLNa) has also been suggested to be involved in the localisation of SK1 to lamellipodia in melanoma cells in response to Heregulin (Maceyka et al., 2008). However, it appears likely that this protein may simply link SK1 to the S1P receptor at this site. In addition, it is yet to be determined whether a conserved role for this protein exists in other cells, and upon other agonist stimulations. Thus, the actual molecular mechanism mediating translocation of SK1 to the plasma membrane has remained unresolved.

In this study, I have identified the CaM-related protein calcium and integrin binding protein 1 (CIB1) as a SK1-interacting protein. I show for the first time that CIB1 functions like a Ca^{2+}-myristoyl switch protein and is responsible for mediating the translocation of SK1 from the cytoplasm to the plasma membrane. I also demonstrate that by modulating SK1 translocation, CIB1 mediates the downstream anti-apoptotic effects associated with SK1 signalling. Finally, I demonstrate that overexpression of CIB1 induces neoplastic transformation in cells in an SK-dependent manner, and furthermore, a dominant-negative CIB1 can block neoplastic transformation induced by constitutively active V12-H-Ras.

3.3 Results and Discussion

3.3.1 CIB1 is a SK1-Interacting Protein

In an attempt to identify candidate proteins that may associate with and regulate SK1 a yeast two-hybrid screen was performed with full-length human SK1 as bait (Zebol et al., 2009). One SK1-interacting protein identified by this screen was CIB1 (also known as calmyrin, and kinase-interacting protein, KIP), a 191 amino acid Ca^{2+} binding protein most similar in sequence to CaM and calcineurin B (54% and 57% amino acid sequence similarity, respectively) (Gentry et al., 2005).

To initially examine the interaction between CIB1 and SK1 in mammalian cells, co-immunoprecipitation experiments were performed using cell lysates from HEK293T cells expressing FLAG-tagged SK1 and HA-tagged CIB1. The presence of SK1 in the anti-HA (CIB1) immunocomplexes supported the existence of the CIB1-SK1 interaction in cells (Figure 3.1A). To further confirm this interaction with endogenous SK1, *in vitro* pull down

experiments using GST-CIB1 were performed using cell lysates from untransfected DU145 cells. GST-CIB1 specifically associated with endogenous SK1 from these lysates (Figure 3.1B), supporting a physiological interaction between SK1 and CIB1. To further confirm a physiological interaction between endogenous SK1 and CIB1, co-immunoprecipitation experiments were performed from MCF-7 cell lysates using anti-SK1 and anti-CIB1 antibodies. Low but reproducibly detectable levels of CIB1 were present in the anti-SK1 immunocomplexes (Figure 3.1C), demonstrating an endogenous interaction between these two proteins. While the anti-SK1 antibody showed some non-specific bands in immunoblots, it was able to immunoprecipitate detectable levels of SK1. In contrast, the anti-CIB1 antibody appeared quite specific, displaying a single band in cell lysates and showed no reactivity to the CIB1-related proteins, CIB2-CIB4 (data not shown). The detectable levels of CIB1 present in the anti-SK1 immunocomplexes may be increased with the development of more specific and higher affinity anti-SK1 antibodies.

The crystal structure of CIB1 has been resolved to reveal a compact 22 kDa α-helical protein composed of closely associated globular *N*- and *C*-terminal domains, each comprised of two EF-hands and separated by a flexible linker (Gentry et al., 2005). This structure is similar to both CaM and calcineurin B, as well as the EF-hand containing neuronal Ca^{2+} sensor (NCS) family of proteins (Gentry et al., 2005). While the two *N*-terminal EF-hands of CIB1 (EF1 and EF2) do not bind metal ions, the two *C*-terminal EF-hands (EF3 and EF4) bind Ca^{2+} in a sequential manner with affinities of 1.9 and 0.54 μM respectively (Yamniuk et al., 2004). These values are similar to that seen with NCS proteins, enabling the binding of Ca^{2+} at concentrations just above basal levels (Gentry et al., 2005). Notably, these affinities for Ca^{2+} are approximately 10-fold higher than that of CaM (Burgoyne and Weiss, 2001). Ca^{2+} binding elicits a substantial conformational change in CIB1, enabling Ca^{2+}-specific interactions with a number of its interacting proteins (Yamniuk et al., 2004).

To investigate the Ca^{2+} dependence of the CIB1-SK1 interaction, *in vitro* pull down experiments using GST-CIB1 and recombinant SK1 were performed in the presence of Ca^{2+}, Mg^{2+} or EGTA. GST-CIB1 was only able to interact with SK1 in the presence of Ca^{2+} (Figure 3.2A). Although preferentially binding Ca^{2+}, EF3 of CIB1 has also been described as a low-affinity Mg^{2+} binding site, with Mg^{2+} binding enabling the interaction of CIB1 with α_{IIb} integrin cytoplasmic domain peptides, albeit through a thermodynamically distinct

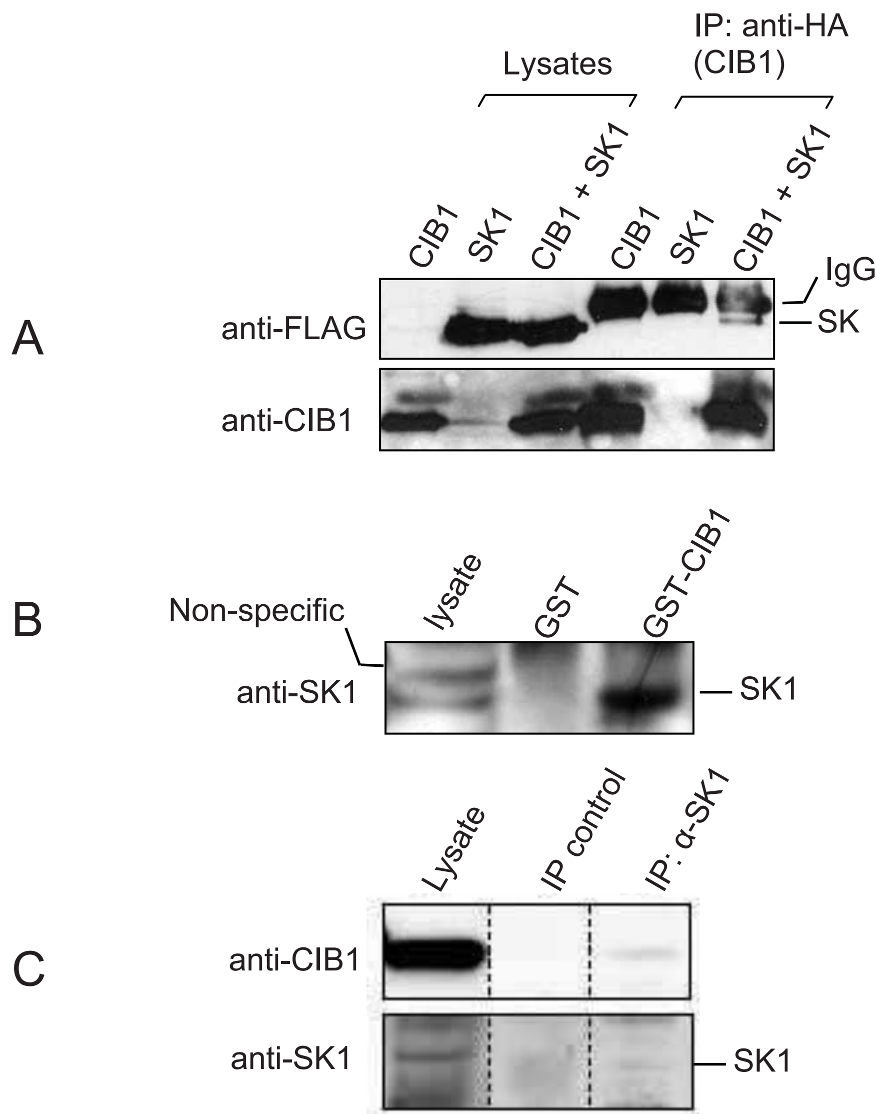


Figure 3.1. CIB1 interacts with SK1

(A) SK1 association with CIB1 was examined by coimmunoprecipitation using lysates from HEK293T cells expressing HA-tagged CIB1 and FLAG-tagged SK1 either individually or together. Expression of these constructs was confirmed via Western Blot (lysate). CIB1 was immunoprecitated via its HA tag and associated SK1 detected by Western blot with anti-FLAG antibodies. IgG is the heavy chain of the anti-HA antibody used in the immunoprecipitation.

(B) Recombinant GST or GST-CIB1 was incubated with cell lysates from untransfected DU145 cells (lysate). Endogenous SK1 pulled-down by GST-CIB1 but not GST alone was detected using anti-SK1 antibodies via Western Blot.

(C) To demonstrate an interaction between endogenous CIB1 and SK1, endogenous SK1 was immunoprecipitated from cell lysates of MCF-7 cells (lysate) using anti-SK1 antibodies and protein A MicroBeads. Lysate containing protein A MicroBeads but no anti-SK1 antibodies was used as a negative control (IP control). CIB1 associated with the anti-SK1 immunocomplexes was detected via Western blot. Dividing lines indicate where lanes from the same Western blot have been spliced together to simplify viewing.

Data are representative of four independent experiments.

manner (Yamniuk and Vogel, 2005). Mg^{2+} however, did not enable the CIB1-SK1 interaction. This was not entirely unexpected as previous NMR spectroscopy studies have revealed subtle structural differences between Mg^{2+}- and Ca^{2+}-bound CIB1 which may facilitate differential binding specificity (Yamniuk and Vogel, 2005). As Mg^{2+} is present in millimolar concentrations inside the cell, it has been suggested that under basal conditions, EF3 is constitutively occupied by Mg^{2+} (Yamniuk et al., 2004; Yamniuk and Vogel, 2005). Increased cytosolic Ca^{2+} levels are predicted to cause the low affinity binding of Mg^{2+} to be displaced by its higher affinity association for Ca^{2+} (Yamniuk et al., 2004). Thus, it is likely that CIB1 interacts with SK1 only upon a rise in Ca^{2+} concentration in the cytoplasm, whereby the EF-hands of CIB1 are occupied only by Ca^{2+}.

To determine the Ca^{2+} concentration dependence of the CIB1-SK1 interaction, further *in vitro* pull down experiments were performed using GST-CIB1 and recombinant SK1 in the presence of 0.5 μM to 2 mM $CaCl_2$. While no interaction was observed in the absence Ca^{2+}, GST-CIB1 interacted with SK1 at as low as 0.5 μM Ca^{2+} (Figure 3.2B). The CIB1-SK1 interaction appeared to be strongest around 0.5 and 2 μM Ca^{2+}, which corresponds well with both physiological cytosolic calcium concentrations (Berridge et al., 2000), and the binding affinities of EF-III and EF-IV for Ca^{2+} (1.9 and 0.5 μM, respectively) (Yamniuk et al., 2004). Thus, it is likely that at least EF-IV of CIB1 is required to be occupied by Ca^{2+} to enable the CIB1-SK1 interaction. Interestingly, at higher Ca^{2+} concentrations, particularly evident at 2 mM, the CIB1-SK1 interaction diminishes (Figure 3.2B). This may be due to the binding of Ca^{2+} into auxiliary binding sites within CIB1 at these high non-physiological Ca^{2+} concentrations. Notably, such binding has previously been shown to alter CIB1 structurally (Yamniuk et al., 2009), and hence this may be responsible for the detrimental affect of high Ca^{2+} on the CIB1-SK1 interaction.

Phosphorylation of human SK1 at Ser225 has been previously described and is responsible for its activation (Pitson et al., 2003) and is also critical for SK1 translocation and associated oncogenesis (Pitson et al., 2005). As phosphorylation is also a well known mechanism for regulation of protein-protein interactions (Wright and Dyson, 2009), I investigated whether the CIB1-SK1 interaction was also dependent upon the phosphorylation status of SK1. Pull down analyses were performed with GST-CIB1 using lysates from HEK293T cells expressing either wildtype SK1 or the non-phosphorylated variant $SK1^{S225A}$. GST-CIB1

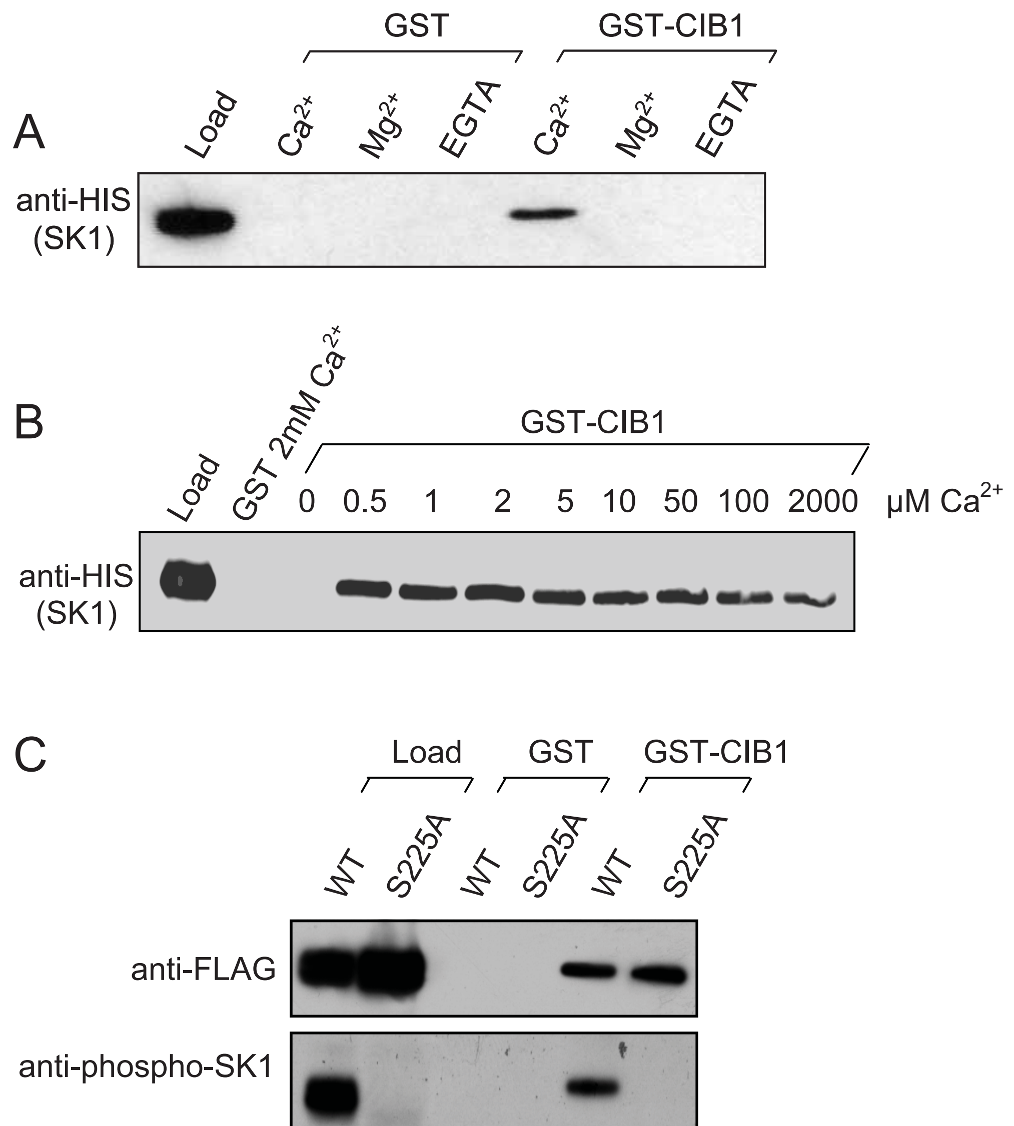


Figure 3.2. Effect of metal ions and SK1 phosphorylation on the CIB1-SK1 interaction

(A) The effect of metal ions on the binding of SK1 to CIB1 was examined using recombinant GST-CIB1 to bind recombinant 6xHis-tagged SK1 (load) in the presence of 2mM $CaCl_2$, $MgCl_2$ or EGTA. Binding specificity was confirmed by the absence of any SK1 bound to GST alone. GST-CIB1 bound to SK1 only in the presence of Ca^{2+}.

(B) The Ca^{2+} concentration dependence of this interaction was determined by performing similar pulldowns with GST-CIB1 and recombinant 6xHis-tagged SK1 under the range of indicated $CaCl_2$ concentrations.

(C) The ability of CIB1 to bind non-phosphorylated SK1 was tested using recombinant GST-CIB1 and lysates from HEK293T cells expressing either wildtype SK1 or $SK1^{S225A}$ (Loads) . Total SK1 was detected using anti-FLAG antibodies, while phospho-SK1 was detected using anti-phospho-SK1 antibodies. Binding specificity was confirmed by the absence of any SK1 bound to GST alone.

Data are representative of three experiments.

was able to bind both wildtype SK1 and SK1^{S225A}, with the intensities of these interactions appearing very similar via Western blot. The overexpression of wild type SK1 in cells gives rise to both phosphorylated and non-phosphorylated protein. Importantly, the presence of phosphorylated SK1 in the GST-CIB1 pull down using lysate expressing wild type SK1 confirmed that GST-CIB1 was able to bind both phosphorylated and non-phosphorylated SK1 (Figure 3.2C). This suggests this interaction is not regulated by phosphorylation of this enzyme.

3.3.2 CIB1 Interacts at the 'CaM-binding site' of SK1

In previous studies the 'CaM-binding site' in SK1 was identified as a critical regulator of the translocation of SK1 to the plasma membrane (Sutherland et al., 2006). As well as sharing considerable sequence and some structural similarity, both CIB1 and CaM appear to target analogous α-helical hydrophobic regions on partner proteins (Barry et al., 2002). Given these similarities, I investigated whether the 'CaM-binding site' on SK1 also mediates the interaction with CIB1.

Pull down analyses were performed with GST-CIB1 using lysates from HEK-293T cells expressing either wild type SK1 or an SK1 variant containing mutations in the 'CaM-binding region' (SK1$^{F197A/L198Q}$) that block its *in vitro* interaction with CaM (Sutherland et al., 2006). Mutation of the 'CaM-binding site' of SK1 largely inhibited its ability to interact with CIB1, indicating that like CaM, CIB1 binds to SK1 at this site (Figure 3.3A).

The CaM inhibitor W7 has been shown to block the Ca^{2+}-associated translocation of SK1 to the plasma membrane (Young et al., 2003). Since phorbol esters have been previously shown to induce reliable translocation of SK1 to the plasma membrane (Pitson et al., 2003; Johnson et al., 2002), I examined whether W7 could also block SK1 translocation induced by this agonist. Thus, we examined the effect of W7 on localisation of GFP-SK1 following cell stimulation with PMA. While PMA induced a robust translocation of GFP-SK1 to the plasma membrane in control cells, pretreatment with W7, however, completely blocked this effect (Figure 3.3B).

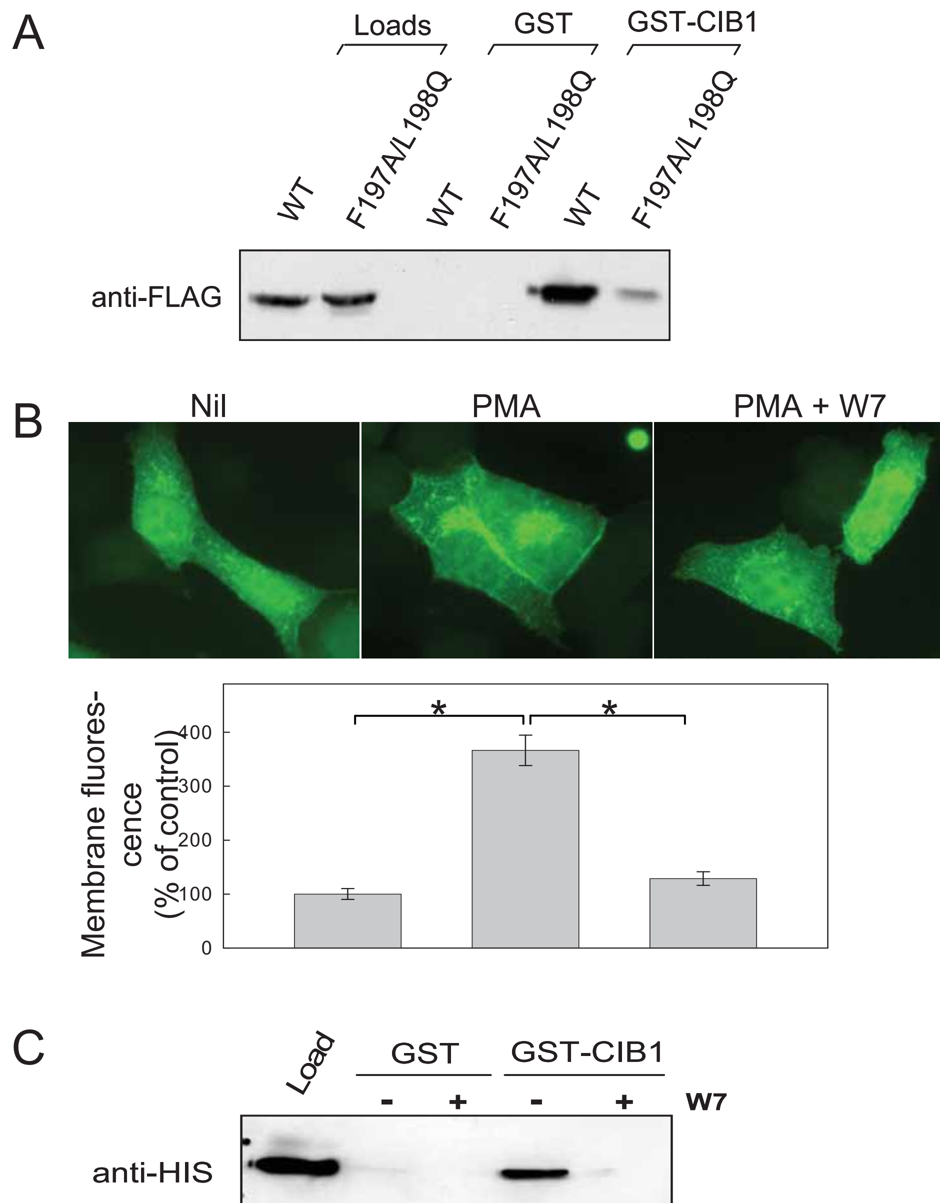


Figure 3.3. CIB1 interacts with SK1 in its 'CaM' binding site

(A) The ability of CIB1 to bind SK1 mutated in the 'CaM-binding site' was examined using recombinant GST-CIB1 and lysates from HEK293T cells expressing FLAG-tagged wildtype SK1 or SK1$^{F197A/L198Q}$. Binding specificity was confirmed by the absence of any SK1 bound to GST alone.

(B) To determine the affect of W7 on phorbol ester-induced SK1 plasma membrane translocation, HeLa cells expressing GFP-SK1 were stimulated with PMA either with or without pretreatment with W7. GFP-SK1 was visualised through fluorescence microscopy. Membrane fluorescence quantitation data represents mean ± S.E from at least 10 cells examined per group. Statistical significance was calculated by an unpaired *t* test. *, $p < 0.0001$.

(C) GST-CIB1 was used to pull-down recombinant 6xHis-tagged SK1 in the presence of 100 µM W7 or DMSO (vehicle control). Binding specificity was confirmed by the absence of any SK1 bound to GST alone.

Data are representative of at least 3 independent experiments.

Although W7 is considered a CaM-specific antagonist (Tanaka, 1988), given the structural similarity between CIB1 and CaM, and the identification that both proteins bind SK1 at the same site, I investigated whether W7 could also be an antagonist for CIB1 and inhibit the CIB1-SK1 interaction *in vitro*. Indeed, I found using pull-down analyses with GST-CIB1 and recombinant SK1 that W7 inhibited the CIB1-SK1 interaction (Figure 3.3C). Thus, the finding that CIB1 interacts with the site on SK1 critical for its translocation, together with this inhibition of the CIB1-SK1 interaction by W7 suggested that CIB1 was a likely candidate for mediating the agonist-induced SK1 translocation.

3.3.3 CIB1 Acts like a Ca^{2+}-Myristoyl Switch Protein

Since its initial discovery in mediating integrin signalling by the platelet-specific integrin $\alpha_{IIb}\beta_3$ (Naik and Naik, 2003b), CIB1 has been shown to be widely expressed in human tissues, and interact with a number of other proteins. These include focal adhesion kinase (Naik and Naik, 2003a), p21-activated kinase 1 (Leisner et al., 2005), two polo-kinases Fnk and Snk (Kauselmann et al., 1999), presenilin 2 (Stabler et al., 1999) and Pax3 (Hollenbach et al., 2002). In each of these cases, CIB1 appears to regulate quite varied signalling outcomes dependent on the target. While the ability of CIB1 to interact with such a diverse range of proteins suggests it may have an extensive role in the regulation of cell signalling, little is known about the mechanisms by which CIB1 exerts its effects. One clue to its potential function came from a study by Stabler et al. (1999) which showed that CIB1 is *N*-terminally myristoylated, leading to the hypothesis that it may act as a Ca^{2+}-myristoyl switch protein. Although this class of proteins is poorly characterized, it is generally understood that the in absence of intracellular Ca^{2+}, the myristoyl group is sequestered into a hydrophobic pocket in the protein. Binding of Ca^{2+} induces a conformation change conferring a dual effect; firstly in enabling the interaction of the protein with target substrates, and secondly to cause the extrusion of the myristoyl group from its original sheltered groove, targeting the protein and any newly associated interacting protein to intracellular membranes (Ames et al., 1997). Hence, this hypothesis provided a potential functional mechanism by which CIB1 may traffic SK1 to the plasma membrane.

To investigate the potential function of CIB1 as a Ca^{2+}-myristoyl switch protein I initially confirmed its *N*-terminal myristoylation. HeLa cells expressing either CIB1 (HA tagged at

the *C*-terminus), HA-CIB1 (tagged at the *N*-terminus to prevent any cotranslational myristoylation) or $CIB1^{G2A}$ (with its myristoylation site, Gly2, mutated to Ala) were metabolically labelled with ^{3}H-myristic acid. Fluorography performed on CIB1 immunoprecipitated from the cell lysates demonstrated that consistent with previous reports (Stabler et al., 1999), CIB1-HA was, indeed, myristoylated (Figure 3.4). Addition of an *N*-terminal HA tag or Gly2→Ala mutation prevented this myristoylation (Figure 3.4).

I then examined the localisation of CIB1 following both an ionomycin- and thapsigargin-induced Ca^{2+} flux. Immunofluorescence performed on cells expressing CIB1 showed this protein translocated from the cytosol to the plasma membrane following ionomycin stimulation (Figure 3.5A). Thapsigargin also induced a weak but significant plasma membrane localisation of CIB1 (Figure 3.5B). These results support the postulated function of CIB1 as a Ca^{2+}-myristoyl switch. It should be noted that these results are in contrast to a previous study which failed to observe a translocation of CIB1 to the plasma membrane upon mobilisation of intracellular Ca^{2+} stores (Blazejczyk et al., 2006). This previous study, however, used a fusion protein of CIB1 with green fluorescent protein (GFP) and thus the presence of GFP may have affected the localisation of this protein.

I further examined the localisation of CIB1 upon SK1 activation using phorbol ester treatment (PMA). Similar to ionomycin stimulation, PMA treatment of cells resulted in a translocation of CIB1 to the plasma membrane which could be prevented by Ca^{2+} chelation with BAPTA/AM (Figure 3.6). This translocation was dependent upon myristoylation of the protein as no cellular relocalisation was observed for the non-myristoylated HA-CIB1. Thus, these findings support the Ca^{2+}-myristoyl switch function of CIB1 and represent an important mechanism by which this protein may exert its regulatory effects on target proteins.

3.3.4 CIB1 Mediates Translocation of SK1 to the Plasma Membrane

To investigate the role of CIB1 in translocation of SK1 to the plasma membrane, I initially examined the relative cellular localisation of CIB1 and SK1 via fluorescence microscopy in cells co-expressing GFP-SK1 and CIB1. In unstimulated cells both proteins were largely

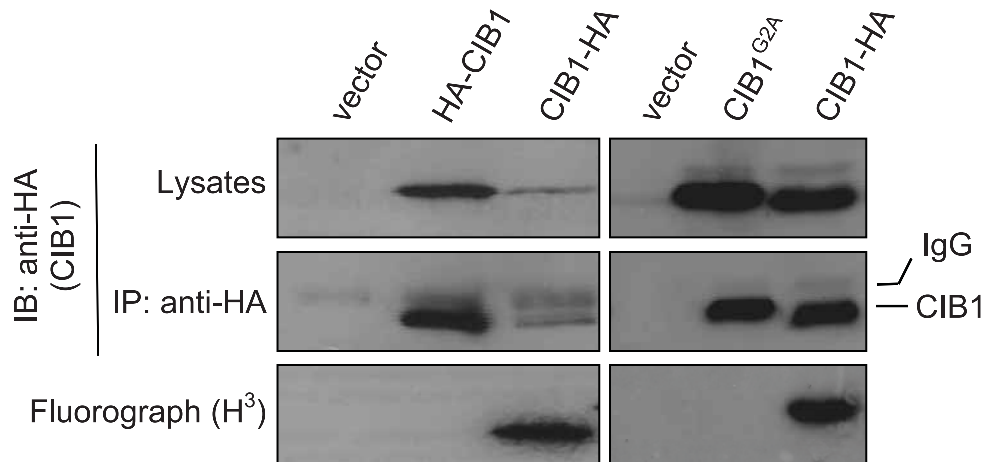


Figure 3.4. CIB1 is myristoylated

The myristoylation status of CIB1 was determined by metabolic labelling of HEK293T cells expressing CIB1 either HA-tagged at the *C*- (CIB1) or *N*-terminus (HA-CIB1) or CIB1^{G2A} with ^{3}H-myristic acid. CIB1 protein was immunoprecipitated from these cell extracts using anti-HA antibodies, proteins separated by SDS-PAGE and the ^{3}H signal detected by fluorography. IgG is the light chain of the anti-HA antibody used in the immunoprecipitation. Data are representative of at least two independent experiments.

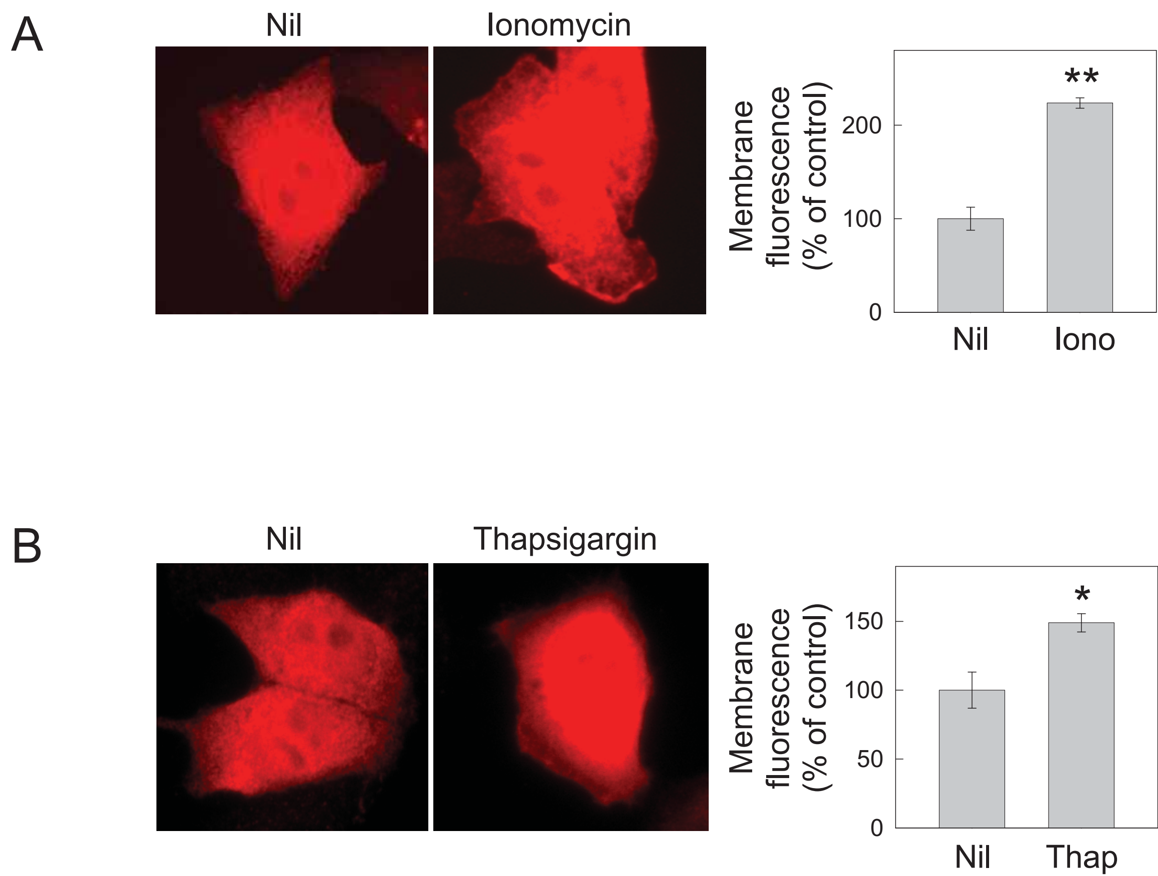
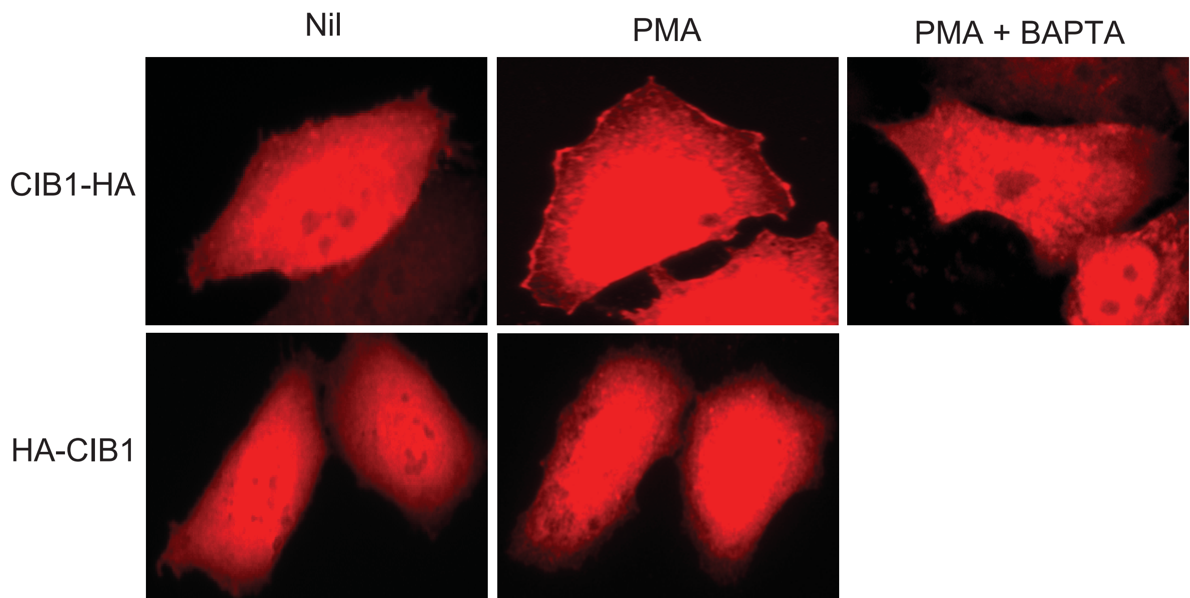


Figure 3.5. CIB1 translocates to the plasma membrane upon induction of a Ca^{2+} flux
Fluorescence microscopy of HeLa cells expressing CIB1 (CIB1-HA) detected by anti-HA antibodies following stimulation with ionomycin (A), or thapsigargin (B). Images are representative of greater than 100 cells examined. Membrane fluorescence quantitation data represent mean ± S.E. from at least 8 cells examined per group. Statistical significance was calculated by an unpaired *t* test. *, $p < 0.02$; **, $p < 0.0001$.



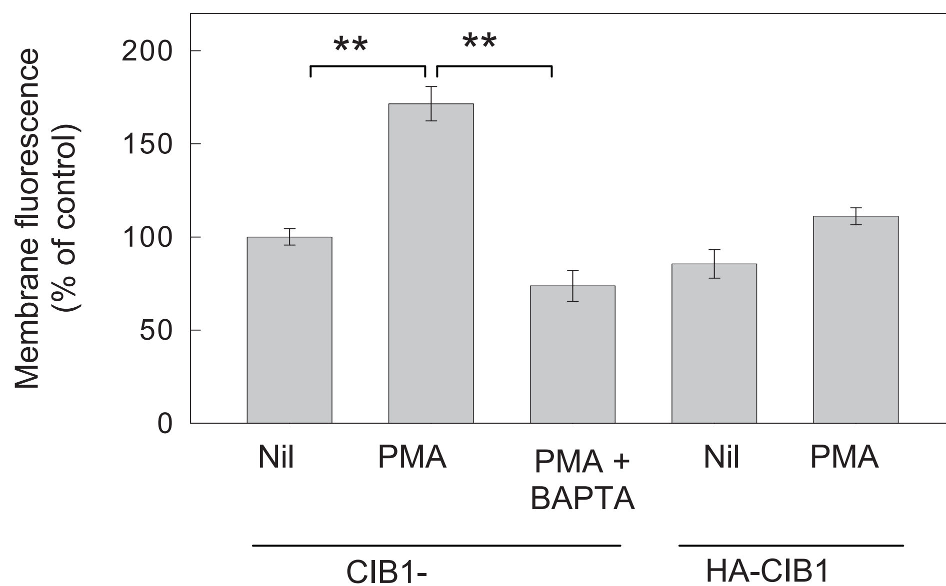


Figure 3.6. CIB1 acts like a Ca^{2+}-myristoyl switch

Fluorescence microscopy of HeLa cells expressing either CIB1 (CIB1-HA) or non-myristoylated CIB1 (HA-CIB1) detected by anti-HA antibodies following stimulation with PMA or BAPTA/AM and PMA. Images are representative of greater than 200 cells examined. Membrane fluorescence quantitation data represent mean ± S.E. from at least 10 cells examined per group. Statistical significance was calculated by an unpaired *t* test. **, $p < 0.0001$.

cytoplasmic, but following PMA stimulation distinct co-localisation of CIB1 and SK1 was observed at the plasma membrane (Figure 3.7).

To determine the requirement of CIB1 for the agonist driven translocation of SK1 I employed siRNA knockdown of CIB1. Following knock-down of CIB1, as monitored by the use of a CIB1 antibody (Figure 3.8A), I examined the localisation of GFP-SK1 in response to PMA stimulation. While GFP-SK1 translocated to the plasma membrane as expected in the control cells, I saw no such relocalisation of SK1 in the CIB1 knock-down cells (Figure 3.8B), suggesting a critical requirement of CIB1 for agonist-induced translocation of SK1. Importantly, in contrast to earlier reports where CIB1 ablation reduced adhesion-induced ERK1/2 activation in endothelial cells (Zayed et al., 2007), ERK1/2 activation in HeLa cells was similar in CIB1 knock-down and control siRNA cells both in unstimulated conditions and following PMA stimulation (Figure 3.9). Hence, the lack of SK1 translocation in CIB1 deficient cells is unlikely to result from disrupted ERK1/2 activation and supports a more direct requirement of CIB1 for SK1 translocation.

Upon activation and translocation of SK1 to the plasma membrane, cellular S1P levels increase (Pitson et al., 2003). Due to the requirement of CIB1 for SK1 translocation, I investigated the effect of CIB1 knock-down on S1P levels following PMA stimulation. While in control cells, PMA stimulation induced a 2-fold increase in S1P levels, this production of S1P was significantly attenuated in CIB1 knock-down cells (Figure 3.10). These results add further support to the role of CIB1 in the PMA-induced translocation of SK1 and the subsequent generation of S1P.

3.3.5 Expression of Non-Myristoylated CIB1 Prevents SK1 Translocation

To further confirm the requirement of CIB1 in the agonist-dependent SK1 translocation, I investigated whether expression of non-myristoylated CIB1 could block the movement of endogenous SK1 to the plasma membrane. Cells expressing HA-CIB1 were treated with PMA, and localisation of this non-myristoylated protein as well as endogenous SK1 was observed by immunofluorescence. Untransfected cells showed a clear relocalisation of endogenous SK1 to the plasma membrane upon PMA stimulation, but this was completely absent in cells expressing HA-CIB1 (Figure 3.11). These results indicate that

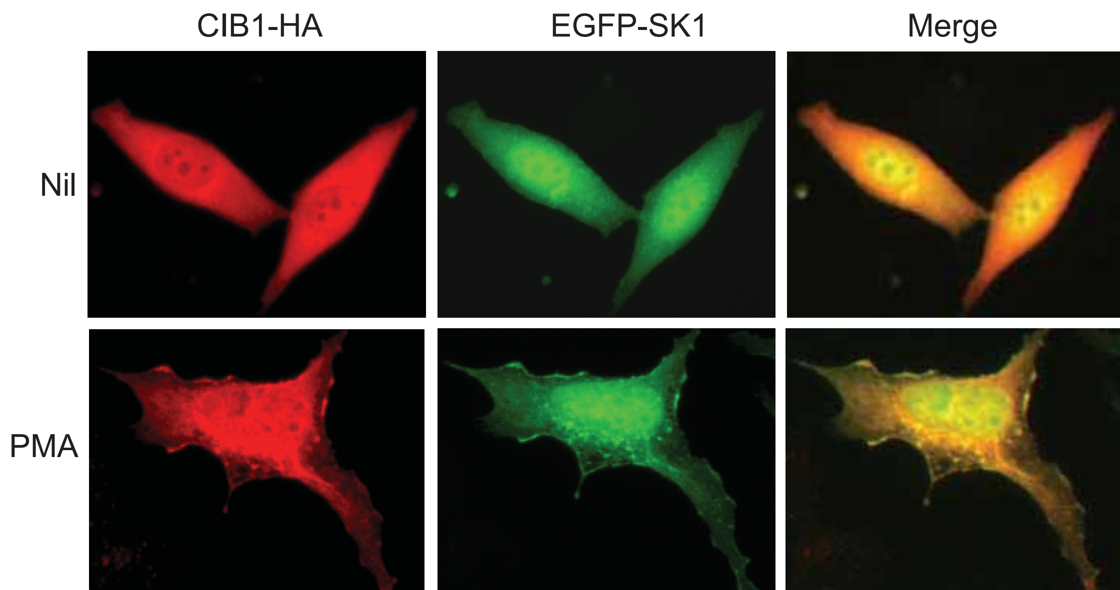


Figure 3.7. CIB1 and SK1 co-localize at the plasma membrane following agonist stimulation

Fluorescence microscopy of HeLa cells co-expressing CIB1 and SK1 following PMA stimulation shows co-localisation of CIB1 (red) and SK1 (green) at the plasma membrane after agonist stimulation. CIB1 was detected using anti-HA antibodies, while SK1 was fused to EGFP.

Images are representative of greater than 100 cells examined.

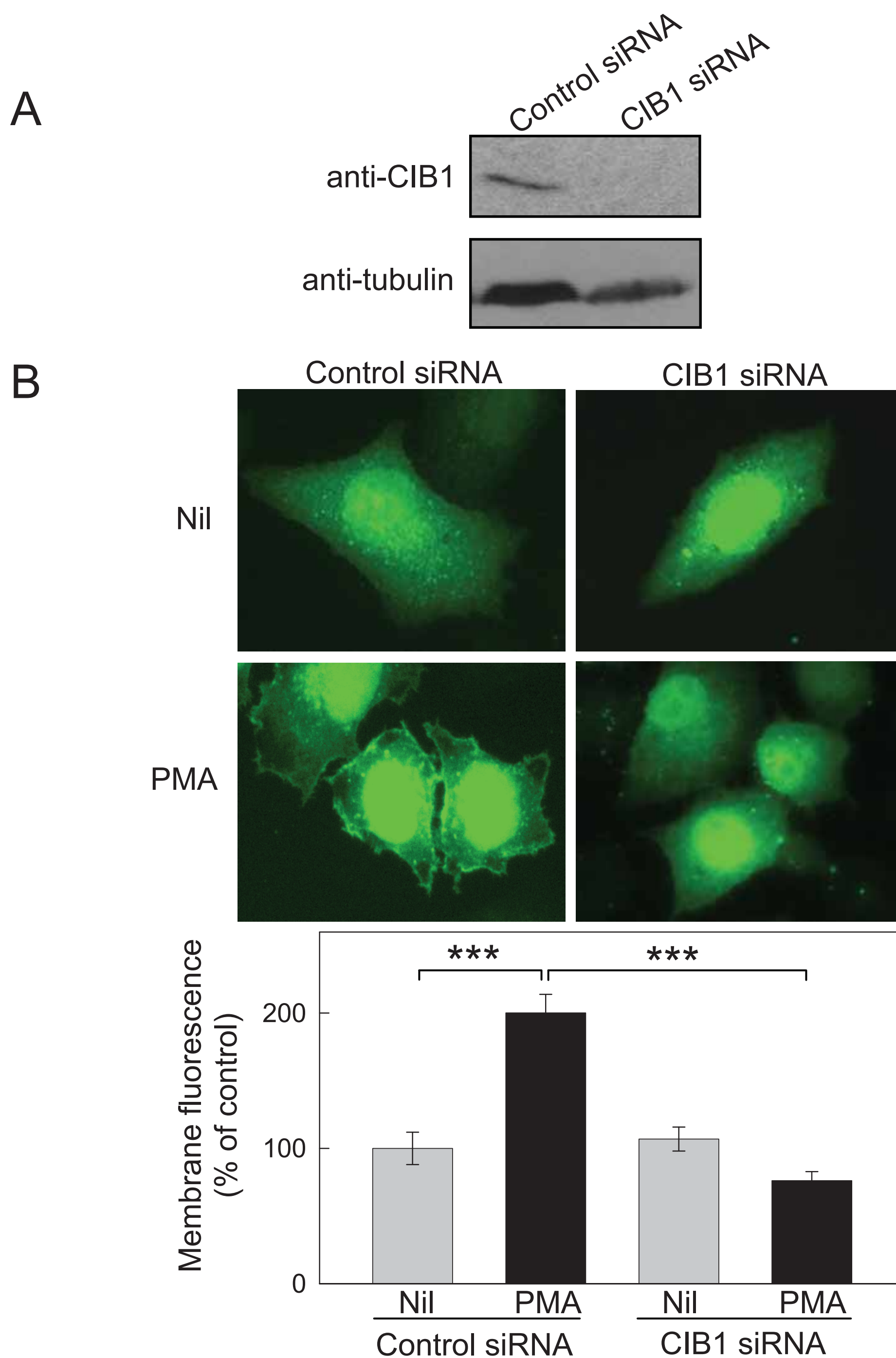


Figure 3.8. CIB1 mediates the agonist-dependent translocation of SK1 to the plasma membrane

(A) Western blot of HeLa cell extracts expressing GFP-SK1 and either a control or CIB1 siRNA demonstrating a knock down of endogenous CIB1 protein. **α**-Tubulin levels show protein loading. (B) Fluorescence microscopy of HeLa cells co-expressing GFP-SK1 (green) and control or CIB1 siRNA with and without PMA stimulation. Experiments were performed with two independent CIB1 siRNAs, each generating comparable results. Membrane fluorescence quantitation data represent means ± S.E. from at least 10 cells examined per group. Statistical significance was calculated by an unpaired *t* test.

Images are representative of greater than 200 cells examined, in three independent experiments.

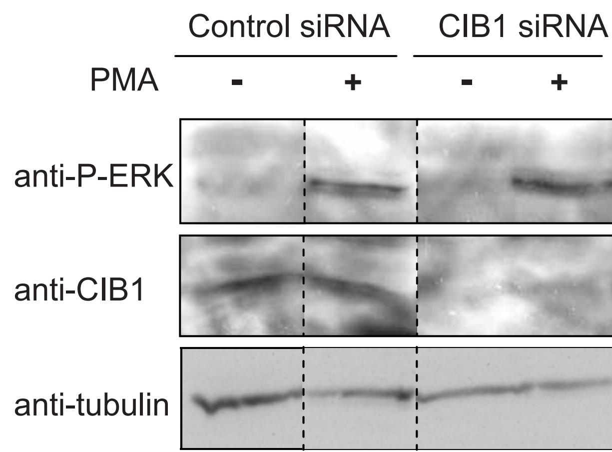


Figure 3.9. Knock-down of CIB1 does not affect ERK activation

(A) ERK1/2 phosphorylation was monitored in either control or CIB1 siRNA transfected cells with or without PMA stimulation by Western Blot. Dividing lines indicate where lanes from the same Western blot have been spliced together to simplify viewing. Data are representative of three experiments.

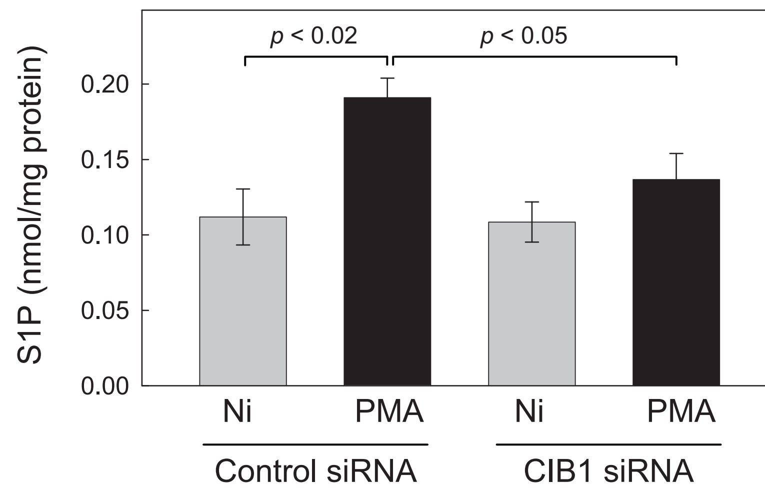


Figure 3.10. CIB1 knock-down inhibits agonist-induced S1P generation

S1P levels in either control or CIB1 siRNA transfected cells with or without PMA stimulation. Data represents the mean ± SD of three independent experiments, with the *p*-value calculated by an unpaired *t*-test.

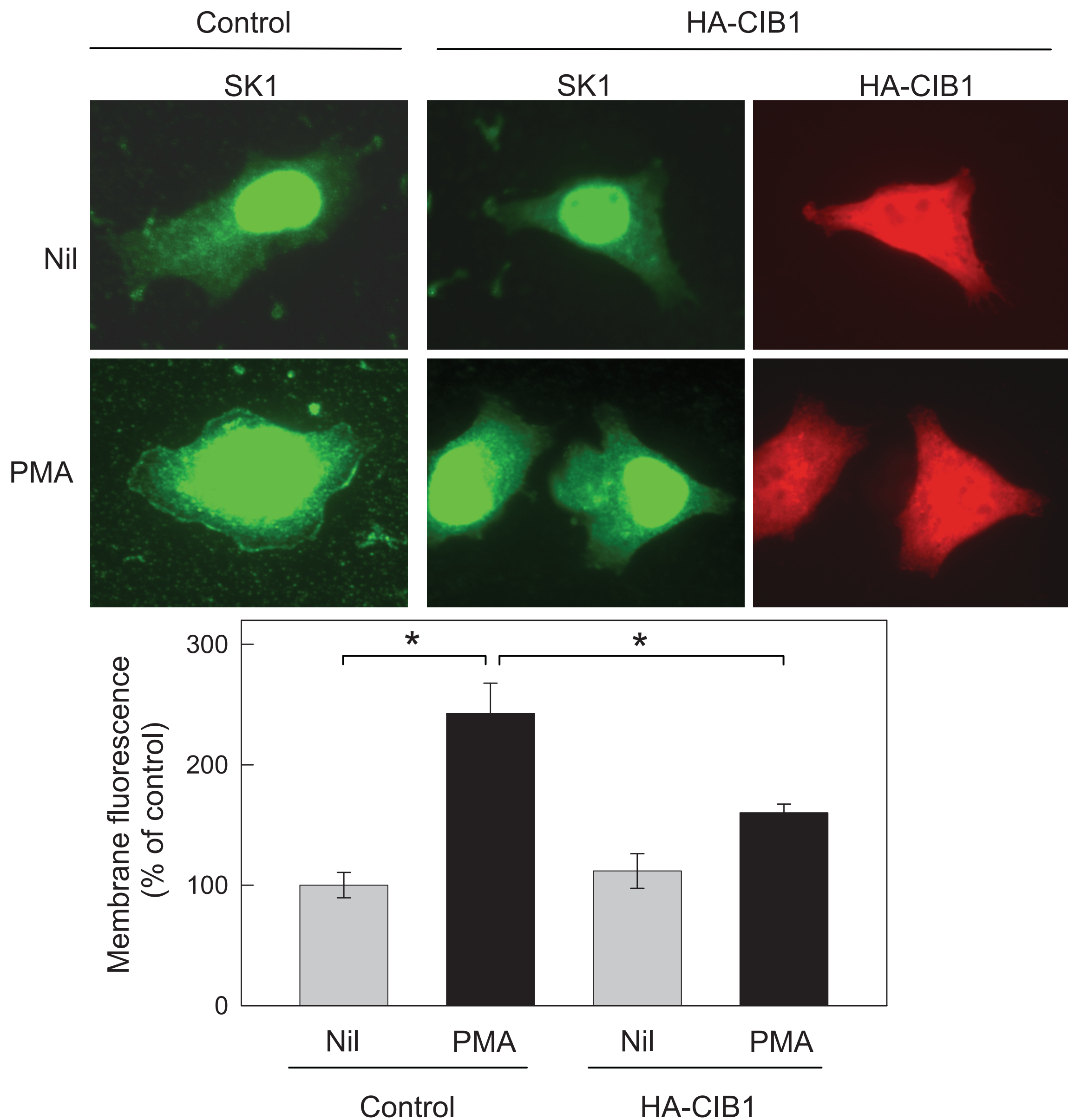


Figure 3.11. Non-myristoylated CIB1 blocks endogenous SK1 translocation

Fluorescence microscopy of either untransfected HeLa cells or cells expressing HA-CIB1 (red) with or without PMA stimulation. Endogenous SK1 (green) was detected using anti-SK1 antibodies. Membrane fluorescence quantitation data represent mean ± S.E. from at least 8 cells examined per group. Statistical significance was calculated by an unpaired *t* test. *, $p < 0.002$.

Images are representative of greater than 200 cells examined.

overexpression of the non-myristoylated CIB1 can act like a ‘dominant-negative’ to block SK1 translocation by endogenous CIB1. Notably, strong nuclear staining of endogenous SK1 was visible in all cells examined. While a nuclear role of SK1 has not yet been described, this localisation is similar to that observed by Johnson and colleagues (2002). As this nuclear localized SK1 did not appear to change following agonist stimulation, it is likely that only the cytoplasmic SK1 relocalises to the plasma membrane.

3.3.6 Knock-Down of CIB1 or Expression of Dominant-Negative CIB1 Enhances Cell Susceptibility to TNF-α-Induced Apoptosis

SK1 activation has been shown to be critical in prevention of TNF-α-induced apoptosis through NF-κB activation (Xia et al., 2002). To investigate the involvement of CIB1-mediated SK1 translocation in this process I examined apoptosis induced by treatment with TNF-α and cycloheximide (CHX) in cells where CIB1 expression was knocked-down by siRNA. In comparison to control cells, the level of apoptosis following treatment with TNF-α and CHX was greatly increased in the CIB1 knock-down cells (Figure 3.12). Notably, this is consistent with previous studies that have shown embryonic fibroblasts from CIB1 knock-out mice display enhanced apoptosis compared to cells from control mice (Yuan et al., 2006b).

To determine whether the myristoylation of CIB1 was required for this anti-apoptotic signalling, I also examined apoptosis induced by treatment with TNF-α and CHX in cells expressing either wild type CIB1 or non-myristoylated CIB1 ($CIB1^{G2A}$). These results showed that while cells expressing wild type CIB1 underwent levels of apoptosis comparable to vector transfected cells, those expressing non-myristoylated CIB1 had an elevated level of apoptosis after TNF-α and CHX treatment comparable to that observed in the CIB1 knock-down cells (Figure 3.13A). To further verify these results, caspase 3/7 activity was measured from the above cell lysates and calculated as caspase 3/7 activity per cell. Surprisingly, no changes in caspase 3/7 activity were observed following TNF-α and CHX treatment in control or CIB1 transfected cells. However, a small but significant increase in activity was observed in $CIB1^{G2A}$ expressing cells (Figure 3.13B). This data not

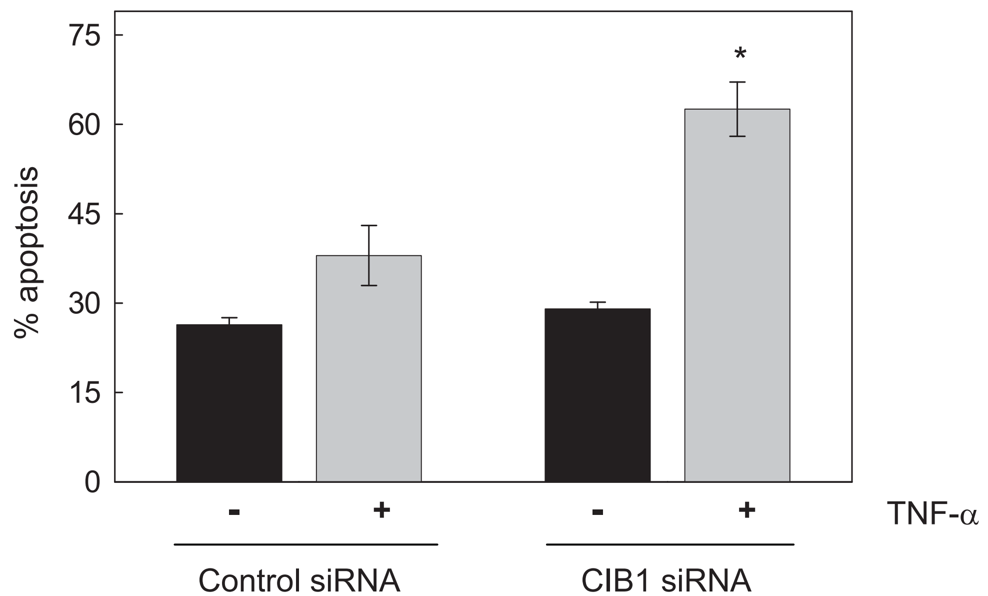


Figure 3.12. CIB1 knock-down enhances cell susceptibility to TNF-α-induced apoptosis

To examine the role of CIB1 in TNF-α mediated cell survival, apoptosis was measured in HeLa cells transfected with either control or CIB1 siRNA and treated with TNF-α and CHX for 22 h. Apoptosis was measured through the percentage of floating cells with ~99% of these floating cells showing positive cell surface staining for the apoptosis marker Annexin V. Data are the mean percentage apoptosis ± SD of three independent experiments. Statistical significance was calculated by an unpaired t-test. * $p < 0.003$

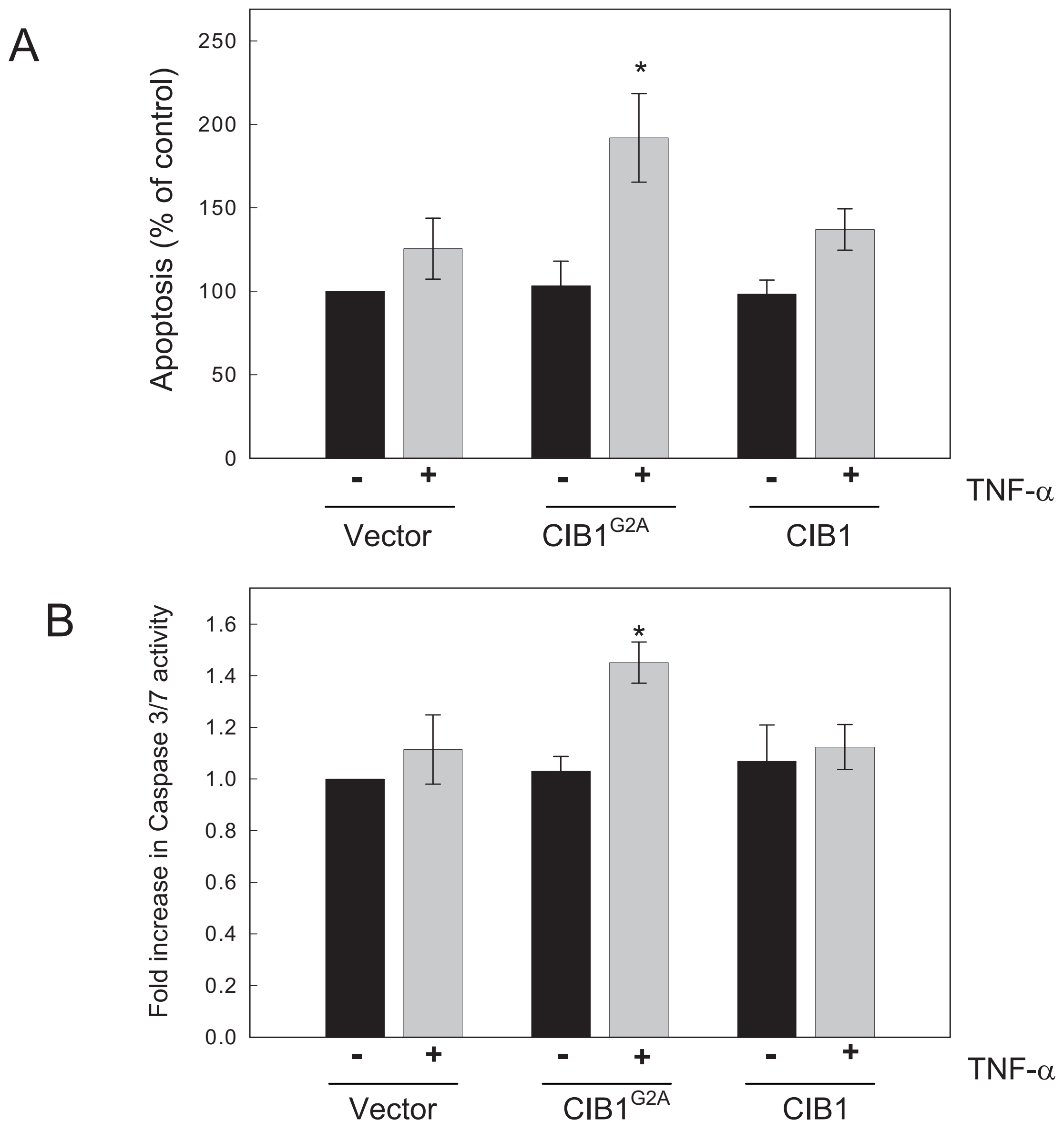


Figure 3.13. Use of a dominant-negative CIB1 enhances cell susceptibility to TNF-α-induced apoptosis

(A) To examine whether the myristoylation of CIB1 was required for its anti-apoptotic signalling, either wild type CIB1 or non-myristoylated CIB1^{G2A} were expressed in HeLa cells and apoptosis measured after TNF-α and CHX for 18 h. Apoptosis was measured through the percentage of floating cells with ~99% of these floating cells showing positive cell surface staining for the apoptosis marker Annexin V. Data are the mean percentage increase ± SD of three independent experiments relative to nil treated vector cells. Statistical significance was calculated by an unpaired t-test. * $p < 0.02$

(B) Caspase 3/7 activity assays were performed on the above cell lysates, with caspase 3/7 activity calculated per cell. Data represents mean increase ± SD of three independent experiments relative to nil treated vector cells. Statistical significance was calculated by an unpaired t-test. * $p < 0.02$

only highlights the necessity of myristoylation for anti-apoptotic signalling by CIB1, but also supports the dominant negative activity of non-myristoylated CIB1 towards SK1.

I next investigated whether this CIB1-associated anti-apoptotic signalling is mediated through NF-κB activation. CIB1 knock-down prevented NF-κB activation following TNF-α stimulation, demonstrated by the degradation of IκBα (Figure 3.14A). This was further confirmed by the use of a NF-κB reporter gene assays that showed TNF-α-induced NF-κB activation was much lower in CIB1 knock-down cells compared to control cells (Figure 3.14B). This data is consistent with previous findings that SK1 activation is essential in this process (Xia et al., 2002) and suggests that CIB1-mediated translocation of SK1 to the plasma membrane is crucial for prevention of TNF-α-induced apoptosis, with this process likely mediated through NF-κB activation.

3.3.7 CIB1 Expression Induces Neoplastic Transformation of NIH3T3 Cells in an SK-dependent Manner.

SK1 translocation to the plasma membrane is essential for its associated oncogenesis (Pitson et al., 2005). Due to the link between CIB1 and this process, I investigated whether expression of CIB1 had the ability to induce neoplastic transformation of cells. Focus formation in NIH3T3 fibroblasts showed that cells stably expressing CIB1 induced numerous foci, indicating a lack of contact inhibition in these cells. Those expressing the myristoyl deficient CIB1 ($CIB1^{G2A}$) however, did not generate foci (Figure 3.15). These results represent the first evidence towards the ability of CIB1 to induce neoplastic transformation. The lack of foci induced by the expression of myristoyl deficient CIB1 demonstrates the importance of this modification and also the Ca^{2+} myristoyl switch behaviour for CIB1. Notably, addition of the SK inhibitor, SK-I, prevented the formation of these foci (Figure 3.15), demonstrating the SK dependence of CIB1's transforming ability and further highlights the oncogenic outcome associated with SK1 translocation.

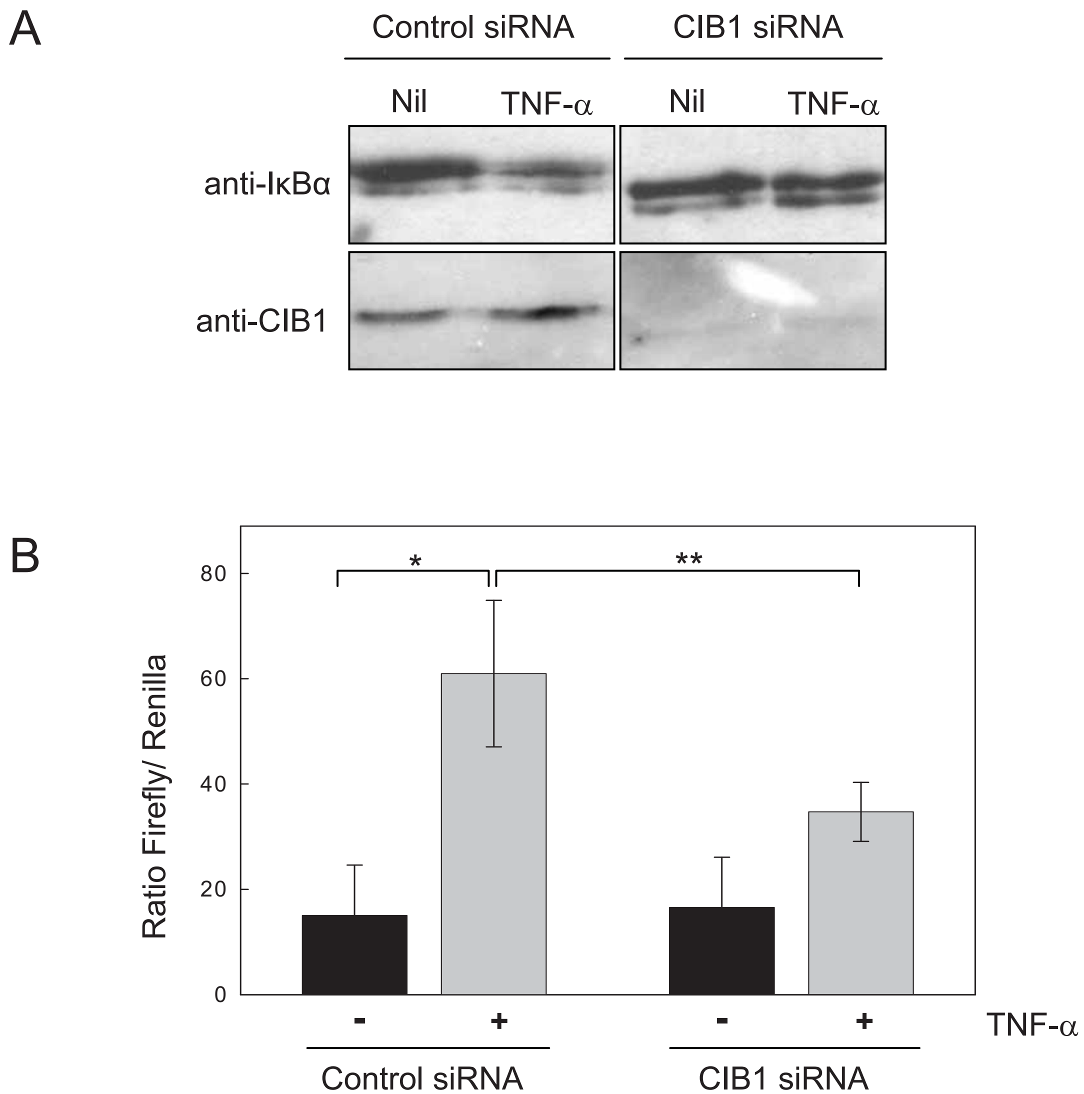
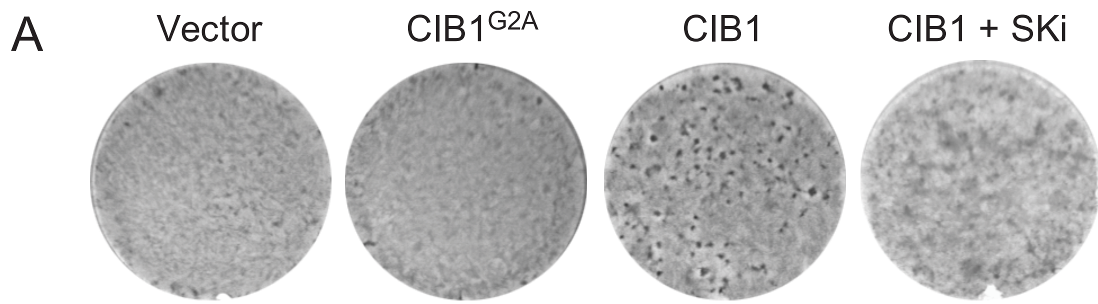


Figure 3.14. Enhanced cell susceptibility to TNF-α-induced apoptosis in CIB1 knock-down cells is mediated through the NFκB pathway

(A) HeLa cells transfected with either control or CIB1 siRNA were stimulated with TNF-α for 30 min and IκB-α levels in cell lysates detected by Western blotting. Data are representative of three experiments.

(B) HeLa cells were transfected with either control or CIB1 siRNA in combination with either vector (pTK81) or pIgKluc, encoding luciferase protein upstream of a region of the IgK promoter, containing several NFκB binding sites. pRL-TK (Renilla) was included in each transfection to standardise transfection efficiency. Two days following transfection, cells were stimulated with 0.5 ng/ml TNF-α for 4 h and a dual-luciferase reporter assay carried out. Firefly and Renilla luciferase activity was standardised and calculated relative to cells expressing a vector control (pTK81). Data is mean ± SD of six independent experiments, with statistical significance was calculated by an unpaired t-test. * $p < 0.0001$. ** $p < 0.002$



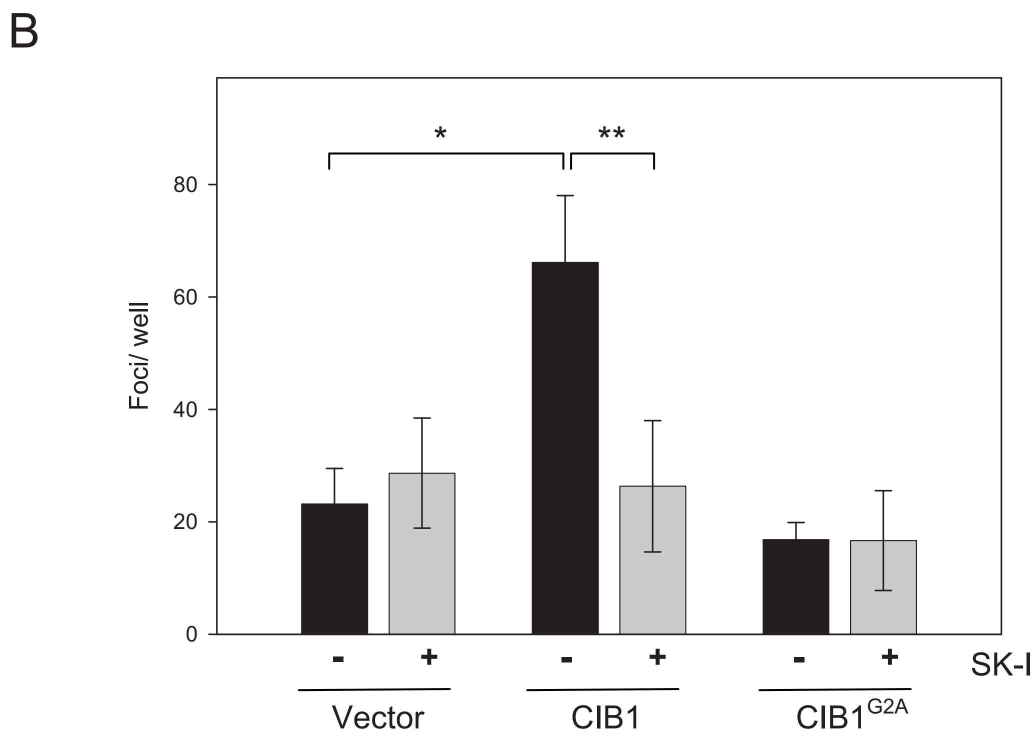


Figure 3.15. CIB1 induces neoplastic transformation of NIH3T3 cells in an SK-dependent manner

The ability of CIB1 overexpression to induce neoplastic transformation was examined by focus formation. NIH3T3 cells stably expressing CIB1, CIB1^{G2A} or empty vector were seeded into 6-well plates and cultured for 3-4 weeks, with media replaced every 2-3 days in the presence or absence of 5 mM SK-I . Foci were scored after fixing with methanol and staining with 0.01% methyl violet.

(A) Representative photographs of stained foci in CIB1 expressing NIH3T3 cells.

(B) Data is mean number of foci per dish ± SD of three independent experiments, with statistical significance calculated by an unpaired t-test. * $p < 0.005$. ** $p < 0.02$

3.3.8 Dominant-Negative CIB1 Inhibits Ras-Induced Neoplastic transformation of NIH3T3 Cells

SK1 activation has been previously shown to have a role in Ras transformation, with chemical inhibitors of SK and a catalytically inactive SK1 mutant ($SK1^{G82D}$) able to reduce Ras transformation (Xia et al., 2000). To examine whether the CIB1-mediated SK1 translocation is also involved in this oncogenesis, I investigated whether the dominant-negative CIB1 ($CIB1^{G2A}$) had an effect on Ras transformation. As expected, when NIH3T3 cells stably expressing CIB1 or vector control cells were transfected with an activated mutant of H-Ras (V12-Ras), both formed foci. NIH3T3 cells expressing the dominant negative CIB1 ($CIB1^{G2A}$) were, however, remarkably resistant to Ras induced transformation (Figure 3.16). These results, combined with the previous findings (Xia et al., 2000) suggest the importance of SK1 translocation in the Ras oncogenic pathway.

3.4 Conclusions and Implications of this Study

Localisation of SK1 to the plasma membrane is crucial for oncogenic signalling by this enzyme. In this study, I have identified that CIB1 mediates the agonist-induced translocation of SK1 to the plasma membrane, and thus established that CIB1 is an important regulator of SK1 function. The identification of the Ca^{2+}-myristoyl switch function of CIB1 is the first elucidation of a mechanism by which CIB1 may mediate its regulatory effects on its various target proteins in cells. We propose a model whereby under basal conditions, the EF-hands of CIB1 are bound by Mg^{2+}, preventing the interaction of CIB1 with SK1. Upon activation of SK1, increases in cytosolic Ca^{2+} concentration have been widely observed (Spiegel and Milstien, 2003a), and this would firstly enable CIB1 to interact with SK1, but also result in the translocation of CIB1 and newly bound SK1 to the plasma membrane. Notably, CIB1 binds to both phosphorylated and non-phosphorylated SK1, despite the observation that non-phosphorylated SK1 does not appear to localize to the plasma membrane. However, as phosphorylated SK1 has been reported to preferentially bind phosphatidylserine (Stahelin et al., 2005), it is possible that both phosphorylated and non-phosphorylated SK1 move to the plasma membrane, but only phosphorylated SK1 is retained at this site via association with phosphatidylserine (Figure 3.17).

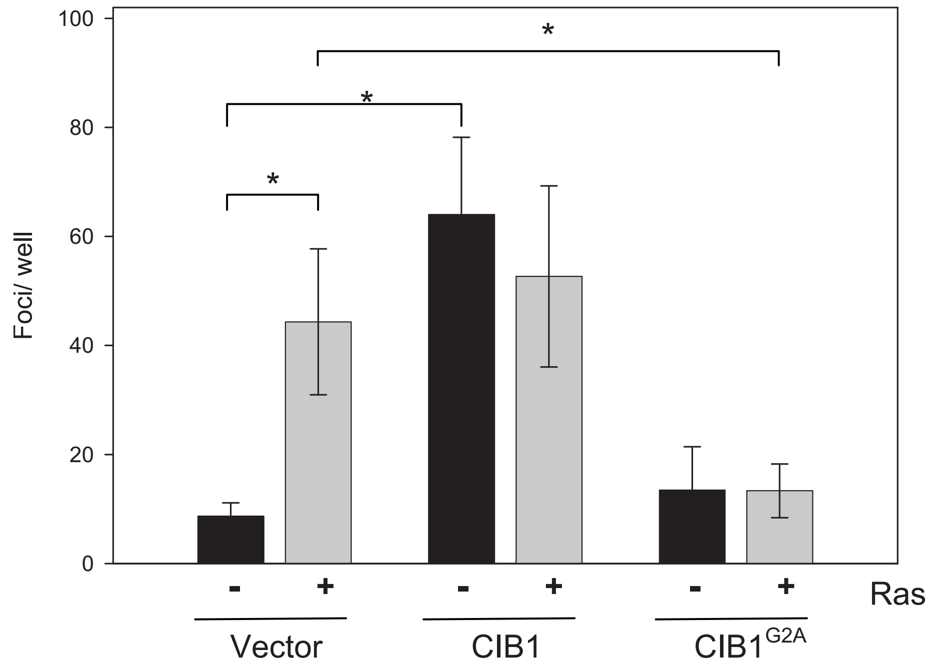


Figure 3.16. Expression of a non-myristoylated dominant-negative CIB1 blocks Ras-induced neoplastic transformation of NIH3T3 cells.

One day after seeding into 6-well plates, NIH3T3 cells stably expressing CIB1, CIB1^{G2A} or empty vector were transfected with V12-Ras and cultured for 3-4 weeks, with media re-placed every 2-3 days. Foci were scored after fixing with methanol and staining with 0.01% methyl violet. Data is mean ± SD of three independent experiments, with statistical significance calculated by an unpaired t-test. * $p < 0.02$

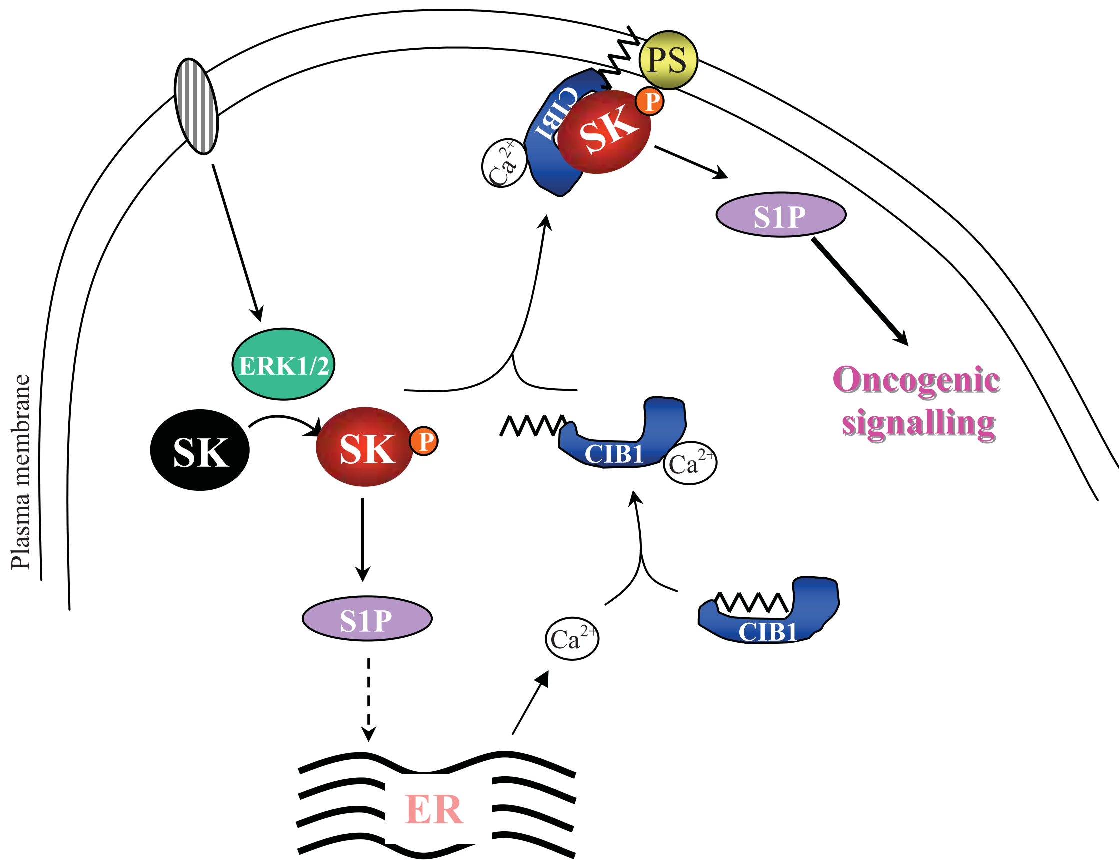


Figure 3.17. Proposed model of phosphorylation– and CIB1-dependant translocation of SK1 to the plasma membrane.

Following agonist stimulation, ERK1/2 phosphorylates SK1 at Ser^{225}, activating the enzyme, resulting in elevated cytoplasmic S1P and an increase in cellular Ca^{2+} proposed through release of this ion from the ER. Ca^{2+}/CIB1 can then interact with SK1 and translocate it to the plasma membrane through its Ca^{2+}-myristoyl switch mechanism. Once at the plasma membrane, retention of phosphorylated SK1 at this site may be mediated via its interaction with plasma membrane-associated phosphatidylserine (*PS*).

The ability of CIB1 to mediate S1P generation and SK1-associated anti-apoptosis illustrates the potential for CIB1 to function in some of the important biological outcomes associated with SK1 signalling. In addition to this anti-apoptotic role, CIB1 has also been shown to mediate cell proliferation, with knockdown of CIB1 reducing proliferation in both mouse heart and lung endothelial cells (Zayed et al., 2007). Again, this is consistent with the pro-proliferative effect of SK1 activation and translocation in cells.

The identification of the ability of CIB1 to induce neoplastic transformation in an SK-dependent manner further signifies the role of CIB1 in the SK1 signalling pathway and is the first evidence towards CIB1 having oncogenic potential of its own. It also further confirms the oncogenic nature of SK1 translocation to the plasma membrane. Further studies are needed to determine the ability of these CIB1 overexpressing cells to lead to tumour formation when injected subcutaneously into mice, with these experiments necessary to confirm the true oncogenic nature of CIB1.

The ability of non-myristoylated CIB1 to act in a 'dominant-negative' fashion in its ability to block both SK1 translocation as well as its pro-survival signalling suggests CIB1 to represent a promising new target to manipulate SK1 signalling in the cell. In addition, the ability of non-myristoylated CIB1 to inhibit Ras-induced oncogenesis point towards CIB1 representing a possible novel target for cancer therapies and suggests SK1 translocation to the plasma membrane to be an integral part of the Ras oncogenic pathway.

One potential issue with quantitation of membrane localisation in this study is the lack of consideration of membrane ruffles. Future experiments should be performed with a membrane marker such as phalloidin to allow for exclusion of these regions in quantitation. However, as eight random membrane locations were quantified per cell, the possible inclusion of some areas of membrane ruffling is unlikely to alter results.

Overall, these findings suggest CIB1 to be an important mediator of some of the biological effects of SK1 activation, and appears to represent a promising target for modulating SK1 signalling outcomes in cells.

Chapter 4: CIB2 Acts as an Endogenous Inhibitor of SK1 Translocation

4.1 Abstract

The Ca^{2+}-myristoyl switch protein CIB1 has been shown to have a critical role in mediating the translocation and subsequent downstream signalling of sphingosine kinase 1 (SK1). CIB2 (Ca^{2+} and integrin binding protein 2) is a homologue of CIB1 about which very little is known. CIB2 shares 59% amino acid similarity with CIB1, and from sequence analysis, appears to have conservation in both its Ca^{2+}-binding EF-hands and hydrophobic-binding pocket with CIB1. Here, I show that like CIB1, CIB2 also interacts with SK1 in the previously identified CIB1 binding region. Unlike CIB1, however, CIB2 interacts with SK1 in a metal ion-independent manner, and despite being myristoylated, does not exhibit Ca^{2+}-myristoyl switch behaviour. Notably, overexpression of wild type CIB2 in HeLa cells inhibited the agonist-induced translocation of endogenous SK1 to the plasma membrane in a comparable manner to that previously observed with a non-myristoylated variant of CIB1. Consistent with these findings, CIB2 overexpression also increased susceptibility of HeLa cells to TNF-α-induced apoptosis. Furthermore, CIB2 overexpression also blocked Ras-induced neoplastic transformation in NIH3T3 cells, which has previously been shown to be at least partially dependent on SK1. Together, these results suggest that CIB2 is an endogenous inhibitor of SK1 translocation and subsequent oncogenic signalling by this enzyme.

4.2 Introduction

The family of calcium and integrin binding proteins (CIBs) is composed of four EF-hand proteins termed CIB1 – CIB4. Of these, CIB1 has been most widely studied, with numerous binding partners identified (reviewed in Yamniuk and Vogel, 2006). CIB1 has also been proposed to have a number of cellular functions, including roles in platelet activation (Yuan et al., 2006b; Tsuboi, 2002), cell survival (Yamniuk and Vogel, 2006), and mediating the signalling of sphingosine kinase 1 (SK1) (Chapter 3). While CIB1 has been quite widely studied, there is currently very little known regarding the functions of the other CIB family members.

CIB2 (also known as kinase interacting protein 2, KIP2) shows 59% amino acid sequence similarity with CIB1 (Figure 1.10) (Gentry et al., 2005) and appears to be ubiquitously expressed in human tissues (Seki et al., 1999). Further sequence analysis suggests that CIB2 possesses the four putative EF-hand Ca^{2+} binding domains (EF1-4) that are also present in CIB1. While only EF3 and EF4 of CIB1 are functional in binding Ca^{2+} it appears likely that CIB2 may bind an extra Ca^{2+} in EF2 due to the presence of more acidic residues in this loop region compared to CIB1 (Gentry et al., 2005). In addition, it appears the overall structure of the hydrophobic binding pocket also remains conserved between CIB1 and CIB2 (Gentry et al., 2005).

Although very little is known about CIB2, recent studies have confirmed it to be a Ca^{2+} binding protein (Hager et al., 2008), and shown that, at least *in vitro*, it can interact with the αIIb cytoplasmic domain (Denofrio et al., 2008). These similarities to CIB1 have suggested the possibility that CIB1 and CIB2 may play similar roles in the cell. Studies have also demonstrated an interaction of CIB2 with the integrin α7Bβ1D, leading to speculation that it may regulate signalling through this integrin in skeletal muscle (Hager et al., 2008). This is similar to one proposed role of CIB1 in the regulation of outside-in signalling through the platelet-specific integrin αIIbβ3 in platelet aggregation (Yamniuk and Vogel, 2006).

I have previously shown that CIB1 has a critical role in mediating translocation of SK1 to the plasma membrane through its function as a Ca^{2+}-myristoyl switch, and thus regulates downstream signalling by this enzyme. Due to the similarities between CIB1 and CIB2, it appeared likely that CIB2 may also interact with SK1 and regulate its localisation and

function in a comparable manner to CIB1. Here I show that, indeed, CIB2 interacts with SK1 in the same binding site as CIB1. However, I discovered that CIB2 does not act as a Ca^{2+}-myristoyl switch, and in complete contrast to CIB1, blocked agonist-induced SK1 translocation and subsequent pro-survival signalling. I also demonstrated that CIB2 expression inhibits neoplastic transformation induced by oncogenic Ras in a comparable manner to that observed following expression of a non-myristoylated variant of CIB1. Together, this suggests a role for CIB2 as an endogenous inhibitor of SK1 translocation and subsequent oncogenic signalling by this enzyme.

4.3 Results and Discussion

4.3.1 CIB2 Interacts with SK1

To initially test the interaction between SK1 and CIB2 in mammalian cells, co-immunoprecipitation experiments were performed using cell lysates from HEK293T cells co-expressing FLAG-tagged SK1 and HA-tagged CIB2. The presence of SK1 in the anti-HA (CIB) immunocomplexes supported the interaction of CIB2 with SK1 (Figure 4.1A). This interaction was further examined by generation of the bacterial expression construct encoding a CIB2 fusion with glutathione *S*-transferase (GST). *In vitro* pull down experiments were then performed using the resultant purified GST-CIB2 protein and lysates from HEK293T cells expressing SK1. GST-CIB2 specifically associated with SK1 from these lysates (Figure 4.1B), supporting an interaction between SK1 and CIB2. Unfortunately, it was not possible to examine an endogenous interaction between these two proteins, due to the current lack of CIB2 antibodies.

CIB1 binds to SK1 in a calcium dependent manner, with Mg^{2+} not able to substitute and facilitate this interaction (Chapter 3). This is despite studies showing that CIB1 is a low-affinity Mg^{2+} binding protein, with Mg^{2+} binding in EF3 eliciting similar structural changes to those observed with Ca^{2+} (Yamniuk and Vogel, 2005). To determine whether CIB2 has a similar metal ion dependence as CIB1, *in vitro* pull down experiments were performed in the presence of Ca^{2+}, Mg^{2+} or EGTA using GST-CIB2 and lysates from HEK293T cells expressing SK1. Results showed that, unlike CIB1, CIB2 was able to interact with SK1 in all conditions tested, suggesting that its interaction is metal ion independent (Figure 4.2).

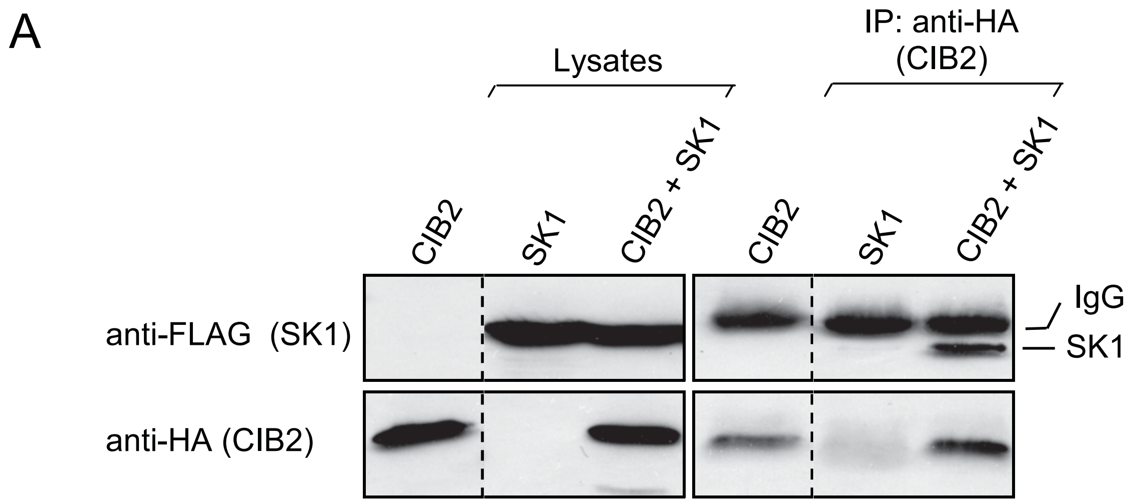


Figure 4.1. CIB2 interacts with SK1

(A) SK1 association with CIB2 was examined by coimmunoprecipitation using lysates from HEK293T cells expressing C-terminally HA-tagged CIB2 and FLAG-tagged SK1 either individually or together. Expression of these constructs was confirmed via Western Blot (lysates). CIB2 was immunoprecitated via its HA tag using anti-HA antibodies and associated SK1 detected by Western blot using anti-FLAG antibodies. SK1 was only detected on the immunoprecipitate from the co-expressing cells. IgG is the heavy chain of the anti-HA antibody used in the immunoprecipitation. Data are representative of 3 experiments.

(B) The association of CIB2 with SK1 was further examined using GST-CIB2 to bind SK1 from HEK293T cells expressing this protein (load). Binding specificity was confirmed by the absence of any SK1 bound to GST alone. Data are representative of 4 experiments. Dividing lines indicate where lanes from the same Western blot have been spliced together to simplify viewing.

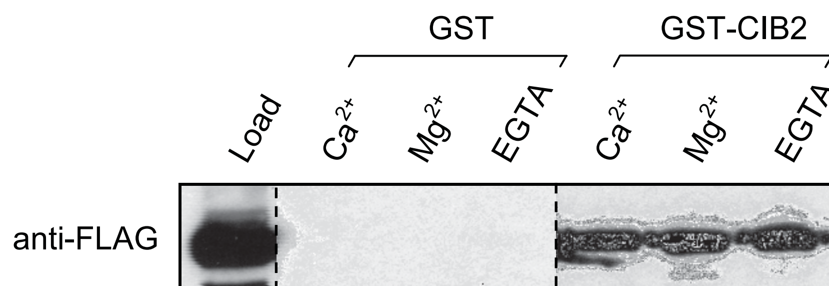


Figure 4.2. CIB2 interacts with SK1 independent of metal ions

The metal ion dependence of the CIB2-SK1 interaction was examined using GST-CIB2 to bind SK1 from HEK293T cells expressing this protein (load), in the presence of 2mM $CaCl_2$, $MgCl_2$ or EGTA. Binding specificity was confirmed by the absence of any SK1 bound to GST alone. Dividing lines indicate where lanes from the same Western blot have been spliced together to simplify viewing. Data are representative of 4 experiments.

This was quite surprising considering the sequence and presumed structural similarity between CIB1 and CIB2 (Gentry et al., 2005) and the known ability of CIB2 to bind Ca^{2+} (Hager et al., 2008).

The binding of metal ions by CIB1 is required to stabilize its structure and also cause it to undergo a significant conformational change (Yamniuk et al., 2004). This is predicted to result in it changing from a 'closed' into a more 'open' structure (Gentry et al., 2005), facilitating interactions with a new range of binding partners in regions of the protein that were previously unexposed. This is supported by the observation that both Ca^{2+}-dependent and -independent binding partners exist for CIB1 (Shock et al., 1999; Blazejczyk et al., 2006). Therefore, it is tempting to speculate that the ability of the CIB2 to bind to SK1 independent of metal ions may be due to it forming a more 'open' structure, similar to Ca^{2+}-CIB1, even in the absence of metal ions. Thus, the subtle variations in its sequence from CIB1 may result in more substantial structural differences in these two proteins. Future elucidation of the structure of CIB2 may reveal the structural basis for these differences in metal ion dependent binding.

4.3.2 CIB2 Interacts with both Phosphorylated and Non-Phosphorylated SK1

As previously described, the phosphorylation of SK1 at Ser225 is an important regulator of SK1 activity, and is critical in SK1-associated oncogenic signalling (Pitson et al., 2005). As phosphorylation is also a well-known regulator of protein-protein interactions, I examined whether mutation of this site would affect the ability of CIB2 to interact with SK1. Pull down analyses were performed with GST-CIB2 and lysates from HEK293T cells expressing either wildtype SK1 or $SK1^{S225A}$. CIB2 was able to bind both wildtype SK1 which is highly phosphorylated, and the non-phosphorylated $SK1^{S225A}$ (Figure 4.3A). Thus, similar to CIB1, it appears that phosphorylation of SK1 does not regulate its interactions with CIB2.

4.3.3 CIB2 Interacts in the CaM/ CIB1 Binding Site of SK1

The amino acid residues Phe197 and Leu198 define a critical region in SK1 for interaction with both CaM (Sutherland et al., 2006) and CIB1 (Chapter 3). As CIB1 mediates the

agonist-induced translocation of SK1 to the plasma membrane, it is not surprising that this site is also a critical regulator of SK1 translocation (Sutherland et al., 2006). Due to the sequence and hypothesized structural similarity between CIB1 and CIB2, I tested whether CIB2 also binds to SK1 in this site. Again, pull-down analyses were performed with GST-CIB2 using lysates from HEK293T cells expressing either wildtype SK1 or an SK1 variant unable to associate with either CaM or CIB1 ($SK1^{F197A/L198Q}$). Indeed, mutation of this CIB1/CaM binding region also attenuated the ability of CIB2 to interact with SK1 (Figure 4.3B). Notably, however, mutation of these residues that block CaM and CIB1 binding only partially inhibits the association of CIB2 with SK1. This suggests that while this site does appear to be important for the interaction of CIB2 with SK1, other residues in SK1 may also be important for this interaction.

4.3.4. CIB2 is Myristoylated

CIB1 is a myristoylated protein (Stabler et al., 1999), utilizing its fatty acid modification to exhibit Ca^{2+}-myristoyl switch behaviour. To determine whether CIB2 also acts in this manner I initially examined its myristoylation. Notably, the *N*-terminus of CIB2 conforms to the myristoylation consensus of ^{1}M-G-X-X-X-S/T (Utsumi et al., 2004). HeLa cells transiently transfected with CIB2 were metabolically labelled with ^{3}H-myristic acid. CIB1 was used as a positive control, while vector and the non-myristoylated HA-CIB1 were used as a negative control. Fluorography was then performed on the CIB proteins immunoprecipitated from the cell lysates. Results demonstrated that indeed CIB2 is myristoylated (Figure 4.4).

4.3.5. CIB2 Does Not Act Like a Ca^{2+}-Myristoyl Switch

As previously described, Ca^{2+} -myristoyl switch proteins are typically characterized by the extrusion of their fatty acid modification from a hydrophobic pocket in the protein into the environment of the cell upon binding Ca^{2+}, enabling their translocation to membranous regions of the cell (Meyer and York, 1999). This is seen with CIB1 by its translocation to the plasma membrane upon stimulation with either phorbol ester (PMA) or the Ca^{2+} ionophore ionomycin (Chapter 3). To examine whether CIB2 also exhibits Ca^{2+}-myristoyl

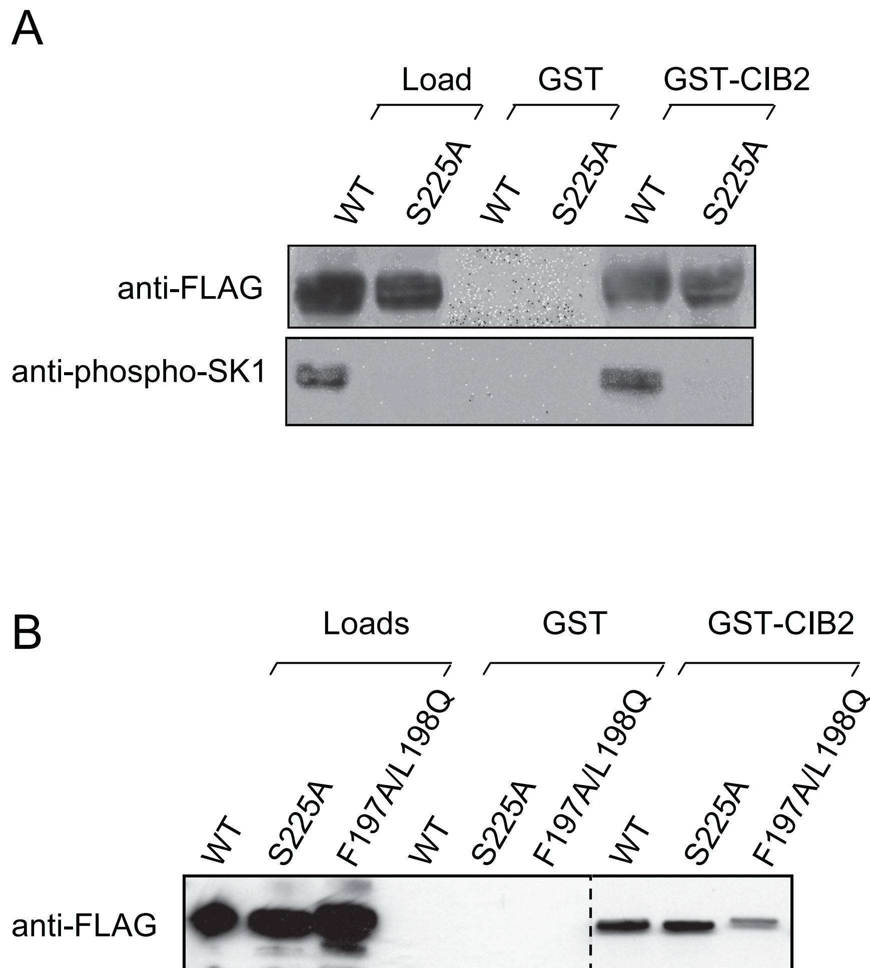


Figure 4.3. Further characterisation of the CIB2-SK1 interaction

(A) The ability of CIB2 to bind both phosphorylated and non-phosphorylated SK1 was tested using recombinant GST-CIB2 and lysates from HEK293T cells expressing either wildtype SK1 (WT) or $SK1^{S225A}$. Total SK1 was detected using anti-FLAG antibodies, while phospho-SK1 was detected using anti-phospho-SK1 antibodies. Binding specificity was confirmed by the absence of any SK1 bound to GST alone.

(B) The ability of CIB2 to bind the CIB1/CaM binding site mutant of SK1 (F197A/L198Q) in comparison to either wildtype SK1 (WT) or non-phosphorylated SK1 was tested using recombinant GST-CIB2 and lysates from HEK293T cells expressing either wildtype SK1 (WT), $SK1^{S225A}$or $SK1^{F197A/L198Q}$. SK1 variants were detected using anti-FLAG antibodies. Binding specificity was confirmed by the absence of any SK1 bound to GST alone. Dividing lines indicate where lanes from the same Western blot have been spliced together to simplify viewing.

Data are indicative of 3 independent experiments.

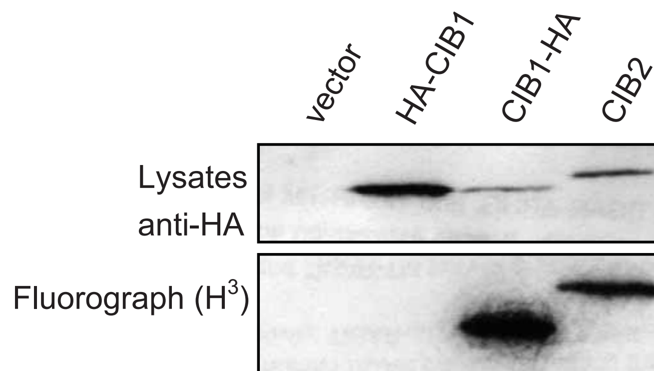


Figure 4.4. CIB2 is myristoylated

The myristoylation status of CIB2 was determined by metabolically labelling HEK293T cells expressing C-terminally HA-tagged CIB2 with ^{3}H-myristic acid. HA-CIB1 (HA-tag at the N-terminus) was used as a negative control, while CIB1-HA (with a HA-tag at the C -terminus) was a positive control. CIB proteins were immunoprecipitated from these cell extracts using anti-HA antibodies, proteins separated by SDS-PAGE and the signal from the incorporated ^{3}H detected by fluorography. Results are representative of 3 experiments.

switch behaviour, I examined its localisation in HeLa cells both with and without stimulation with either PMA or ionomycin. Prior to stimulation, CIB2 was largely cytoplasmic, but also displayed perinuclear staining characteristic of ER localisation. This distribution, however, did not appear to alter upon either PMA or ionomycin stimulation (Figure 4.5). Notably, parallel experiments with CIB1 showed clear translocation of this protein to the plasma membrane, as previously described (Chapter 3). Hence, it appears that CIB2 differs in its functional properties compared with CIB1, and at least in these cells, is unlikely to act like a Ca^{2+}-myristoyl switch.

4.3.6 Overexpression of CIB2 Inhibits Agonist-Induced Translocation of SK1 to the Plasma Membrane

The previous results suggested that CIB2 does not act as a Ca^{2+}-myristoyl switch, and hence was unlikely to function in the same manner as CIB1 in mediating the translocation of SK1 to the plasma membrane. However, since CIB2 associated with SK1 at the same site as CIB1, I examined the possibility that CIB2 may act as an inhibitor of SK1 translocation. Thus, I examined the localisation of endogenous SK1 in cells overexpressing CIB2, with and without exposure to phorbol ester (PMA), a known inducer of SK1 translocation. Similar to that observed previously (Chapter 3), and by others (Johnson et al., 2002), strong nuclear staining of endogenous SK1 in addition to cytoplasmic SK1 was visible in all cells examined. Following PMA stimulation, endogenous SK1 showed clear plasma membrane localisation in control cells as anticipated, demonstrating the effectiveness of the PMA stimulation. In CIB2 expressing cells, however, no such SK1 relocalisation to the plasma membrane was apparent (Figure 4.6). This suggests that CIB2 expression may block the translocation of SK1 to the plasma membrane. One potential rationale to account for this observation may be that the interaction of CIB2 with SK1 in the cytoplasm may restrict the localisation of this enzyme. As CIB1 and CIB2 appear to utilise the same residues on SK1 for their interactions, and CIB2 does not relocalise like a Ca^{2+}-myristoyl switch, it is likely that following agonist stimulation, the interaction of CIB2 with SK1 prevents CIB1 from binding to SK1 and shuttling it to the plasma membrane. Thus, overexpression of CIB2 may act in a similar manner to the ‘dominant negative’ non-myristoylated CIB1 that we previously generated (Chapter 3). This raises the possibility that CIB2 may act as an endogenous inhibitor of agonist induced SK1 translocation.

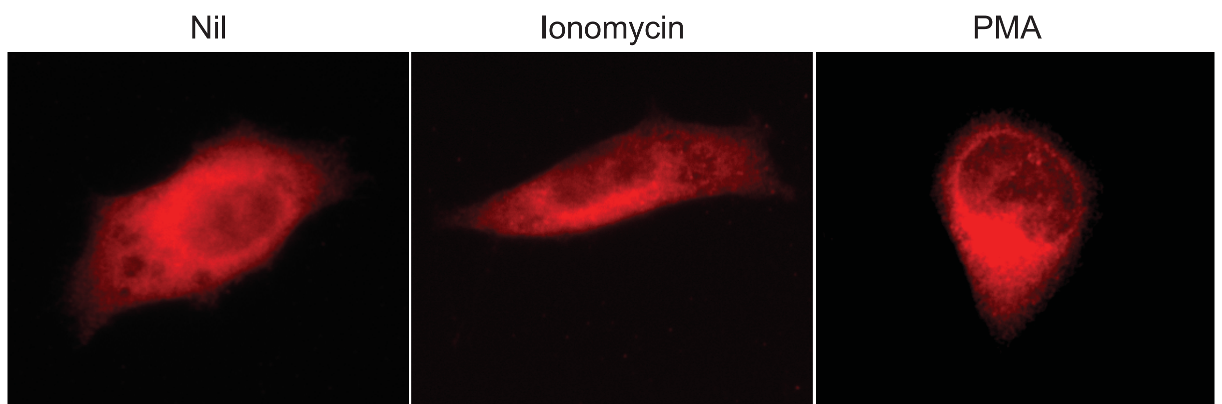


Figure 4.5. CIB2 does not act like a Ca^{2+}-myristoyl switch

Fluorescence microscopy of HeLa cells expressing C-terminally HA-tagged CIB2 detected by anti-HA antibodies following stimulation with 2 μM ionomycin for 2 mins, or 1 μg/ml PMA for 30 mins. Results are representative of over 100 cells examined in 3 experiments.

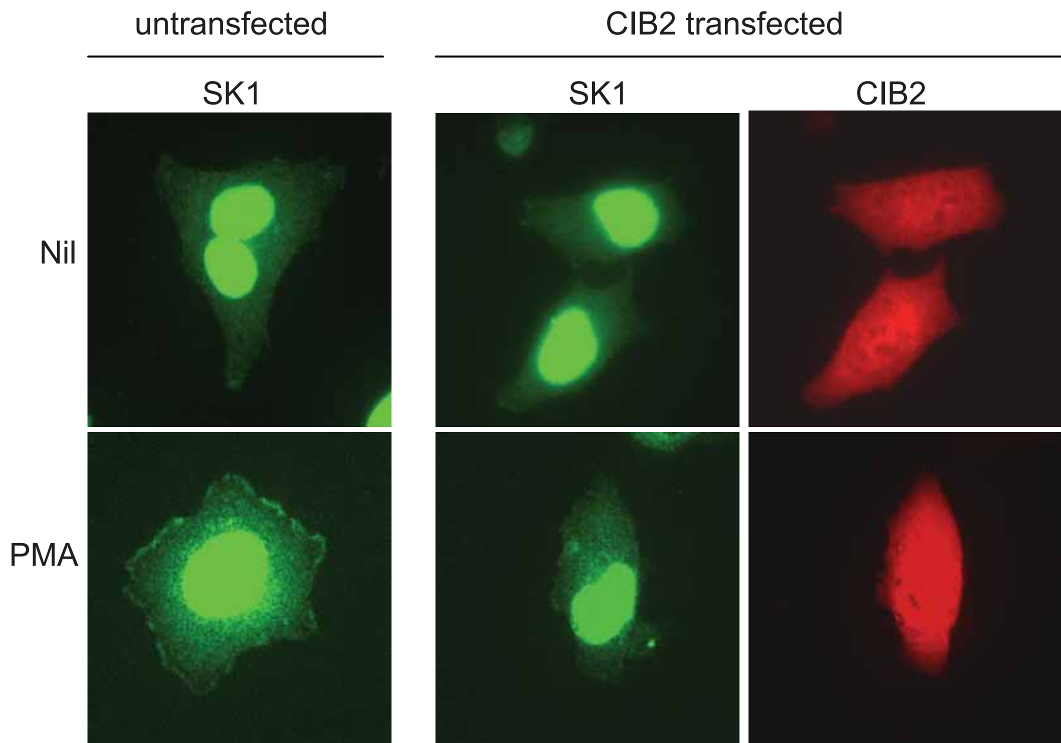


Figure 4.6. Expression of CIB2 blocks agonist-induced translocation of endogenous SK1

Fluorescence microscopy of either untransfected HeLa cells or cells expressing CIB2 (red) with or without phorbol ester (PMA) stimulation. Cells expressing CIB2 (red) were identified using its HA tag, while endogenous SK1 (green) was visualized using anti-SK1 antibodies. Images are representative of 100 cells examined in two independent experiments.

4.3.7 Overexpression of CIB2 Enhances Cell Susceptibility to TNF-α-Induced Apoptosis

I previously showed that blocking SK1 translocation to the plasma membrane through the use of a 'dominant-negative' CIB1 enhances cellular susceptibility to TNF-α induced apoptosis. As CIB2 expression appeared to act in a similar manner to block the agonist-induced translocation of endogenous SK1 to the plasma membrane, I examined whether it too could affect the TNF-α associated pro-survival signalling. To test this, apoptosis was measured in HeLa cells stably expressing either *wild-type* CIB2, CIB1, or a non-myristoylated variant of CIB1 ($CIB1^{G2A}$), following treatment with TNF-α and cycloheximide (CHX) for 18 hours. Results showed that, similar to the 'dominant negative' $CIB1^{G2A}$, cells expressing CIB2 underwent higher levels of apoptosis following TNF-α and CHX treatment compared to either control cells or cells overexpressing CIB1 (Figure 4.7A). To further verify these results, caspase 3/7 activity was measured in lysates from cells treated with TNF-α and CHX as described above. Again, as observed previously (in Chapter 3), very little increase in caspase 3/7 activity was induced by TNF-α/CHX in control cells (Figure 4.7B). However, small but significant increases in caspase 3/7 activity were observed following TNF-α and CHX treatment in the cells overexpressing CIB2 or the 'dominant negative' CIB1. Hence, as well as inhibiting SK1 translocation, CIB2 appears to inhibit this biological outcome associated with SK1 signalling. This adds further support to the possible function of CIB2 as an endogenous inhibitor of SK1 translocation.

4.3.8 Expression of CIB2 Inhibits Ras-Induced Neoplastic Transformation of NIH3T3 Cells

SK1 activation has been previously shown to have a role in Ras-induced neoplastic transformation, with chemical inhibitors of SK and a catalytically inactive SK1 mutant ($SK1^{G82D}$) able to reduce Ras transformation (Xia et al., 2000). I have also established a role for the CIB1-dependent SK1 translocation in Ras oncogenesis, with non-myristoylated 'dominant-negative' CIB1 able to block Ras transformation (Chapter 3). Due to the evidence that CIB2 can inhibit SK1 signalling, I investigated whether it, too, could affect Ras-induced neoplastic transformation. NIH3T3 cells expressing CIB2 alone, oncogenic H-Ras (V12-Ras) alone or coexpressing both proteins were examined for neoplastic

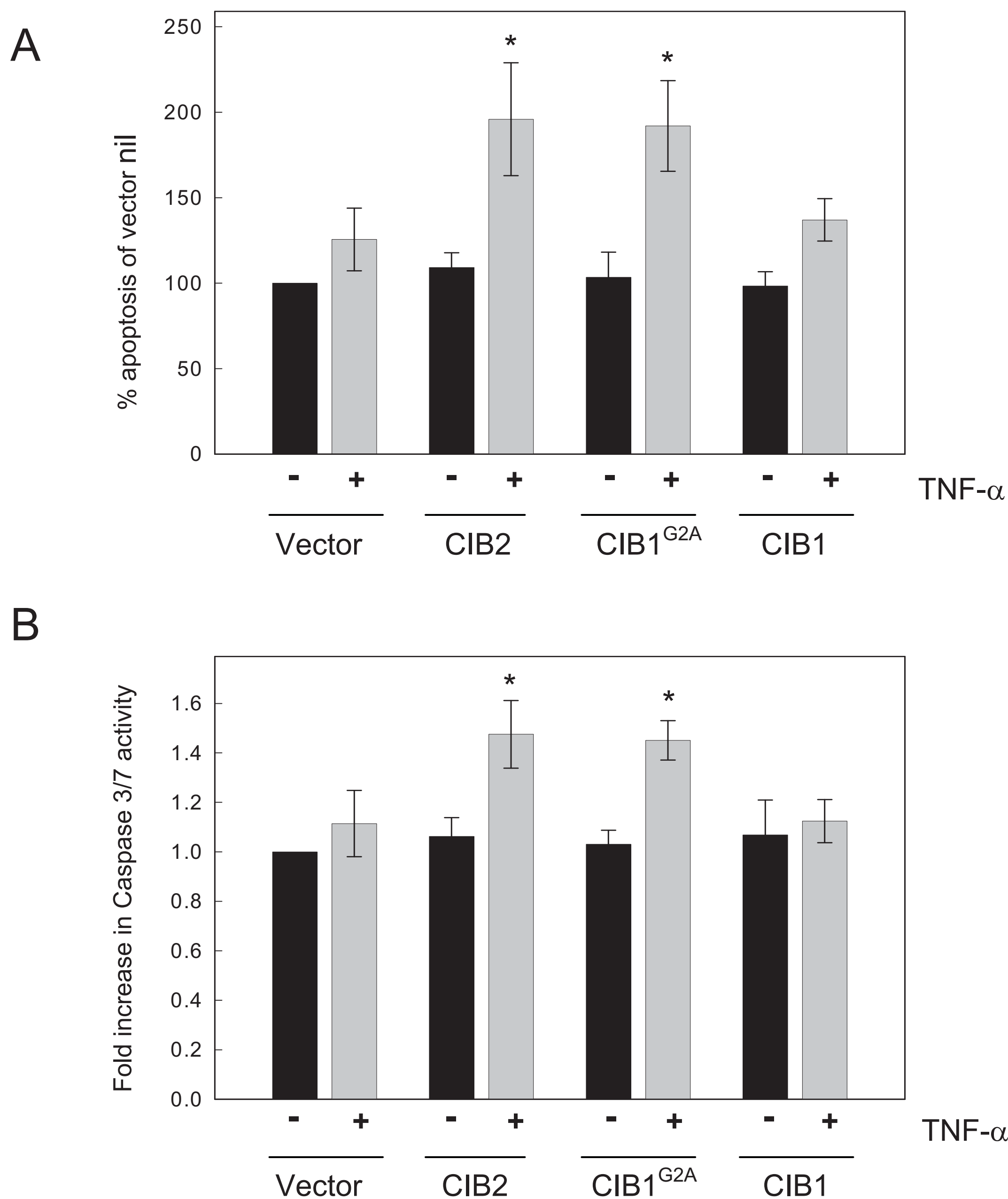


Figure 4.7. CIB2 enhances cell susceptibility to TNF-α-induced apoptosis

(A) To examine whether CIB2 affects the TNF-α stimulated apoptotic/ anti-apoptotic pathway CIB2 was overexpressed in HeLa cells and apoptosis measured after TNF-α and CHX for 18 h. Wild type CIB1 and non-myristoylated $CIB1^{G2A}$ were used as controls. Data are the mean percentage increase ± SD of three independent experiments relative to nil treated vector cells. Statistical significance was calculated by an unpaired t-test. * $p < 0.05$

(B) Caspase 3/7 activity assays were performed on the above cell lysates, with caspase 3/7 activity calculated per cell. Data represents mean increase ± SD of three independent experiments relative to nil treated vector cells. Statistical significance was calculated by an unpaired t-test. * $p < 0.05$

transformation via focus formation assays. As expected due to the opposing roles of CIB1 and CIB2 in SK1 translocation, results demonstrated that, unlike CIB1 (Chapter 3), expression of CIB2 did not transform cells on its own (Figure 4.8). However, while Ras expression alone resulted in substantial focus formation, this was clearly inhibited by co-expression of CIB2. Again, these results further suggest the inhibitory effect of CIB2 on the SK1 signalling pathway.

4.4 Conclusions

The rapid agonist-induced translocation of SK1 to the plasma membrane is essential for its pro-proliferative, anti-apoptotic and oncogenic effects (Pitson et al., 2005). In this study, I have identified that CIB2 can act as an inhibitor of SK1 translocation and subsequent oncogenic signalling. Thus, CIB2 appears to have an opposite function to its closely related protein CIB1, which I have previously shown to mediate the translocation of this enzyme.

Upon agonist stimulation and SK1 activation, cytosolic Ca^{2+} fluxes have been observed to occur in the cell (Spiegel and Milstien, 2003b). The ability of CIB2 to interact with SK1 independent of both Ca^{2+} ions and phosphorylation state suggests a basal interaction between SK1 and CIB2. As CIB2 does not appear to alter its cytosolic localisation upon mobilisation of Ca^{2+} stores, it is feasible that high cellular levels of CIB2 may retain SK1 in the cytoplasm irrespective of upstream signalling events and thus exert its inhibitory effects. The observation that CIB2 does not appear to act like a Ca^{2+} myristoyl switch, despite being myristoylated leads us to question the role for its fatty acid moiety. However, it should be noted that I have only observed the localisation of CIB2 in one cell type, and thus it is possible that it may have a different localisation in other cells.

CIB1 and CIB2 appear to utilise at least some of the same residues in SK1 for interaction. Thus, it is likely that these two isoforms compete for binding to SK1, as both appear to be expressed in a large variety of human tissues (Shock et al., 1999; Seki et al., 1999). Hence it is likely that the interactions of these proteins with SK1 are governed both by binding affinities and their relative cellular expression levels. Thus, it is possible that a basal interaction between CIB2 and SK1 is outcompeted by a higher-affinity interaction with CIB1 upon mobilisation of Ca^{2+} stores, resulting in CIB1 translocating SK1 to the plasma

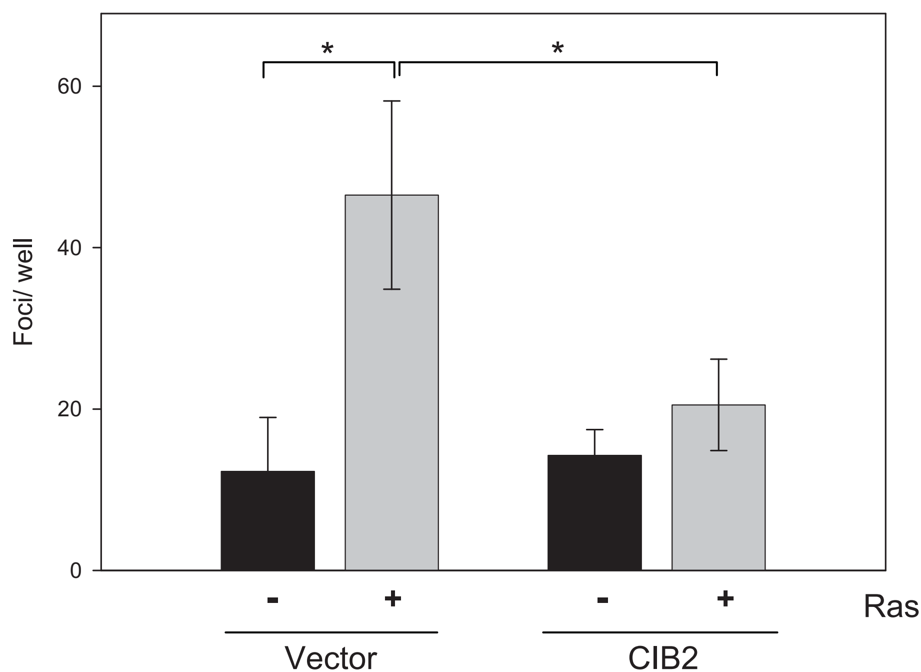


Figure 4.8. CIB2 blocks the Ras oncogenic pathway

One day after seeding into 6-well plates, NIH3T3 cells stably expressing CIB2 or empty vector were transfected with V12-Ras and cultured for 3-4 weeks, with media replaced every 2-3 days. Foci were scored after fixing with methanol and staining with 0.01% methyl violet. Data are mean ± SD of three independent experiments with statistical significance calculated by an unpaired t-test. * $p < 0.01$

membrane and a pro-proliferative, anti-apoptotic and oncogenic outcome. In some situations, however, upregulation of CIB2 expression may occur and prevent this signalling event. Future studies should examine the binding affinities of CIB1 and CIB2 with SK1 to gain a better understanding of the interplay of these proteins in SK1 signalling.

The ability of CIB2 expression to enhance cellular susceptibility to TNF-α induced apoptosis identifies CIB2 as having a biological effect on the signalling outcomes associated with SK1. In addition, its ability to block Ras-induced neoplastic transformation is a significant finding and again highlights the implications of SK1 translocation to the plasma membrane in Ras-mediated oncogenesis.

Chapter 5: Characterisation of CIB3 and CIB4 and their Interactions with SK1

5.1 Abstract

The calcium and integrin binding (CIB) family of proteins consists of four members in humans termed CIB1-4. Of these proteins, CIB1 has been the most widely studied, with multiple interacting partners identified and known roles in platelet activation, cell survival, and mediating the signalling of sphingosine kinase 1 (SK1). I have also described a role for CIB2 in the negative regulation of SK1 signalling, with CIB1 and CIB2 having opposing roles in this pathway. Virtually nothing, however, is known regarding the function of the other CIB family members, CIB3 and CIB4, which share 62% and 64% amino acid sequence similarity with CIB1, respectively. Here, I have begun to characterize these proteins by examining their subcellular localization, ability to interact with SK1 and metal ion dependence. I demonstrate that despite being myristoylated, neither CIB3 nor CIB4 appear to act as Ca^{2+}-myristoyl switches. Both proteins, however, interact with SK1 at the previously identified CIB1 binding site in a manner independent of metal ions. In an attempt to determine the biological outcomes of the interactions of these CIB proteins with SK1, I found that similar to CIB2, expression of CIB4 was able to block the agonist-induced translocation of SK1 to the plasma membrane and inhibit the anti-apoptotic signalling associated with the membrane localization of this enzyme. Quite surprisingly, however, expression of CIB4 induced neoplastic transformation of cells in an SK-independent manner. CIB3 did not appear to have any role in the SK1 signalling pathway.

5.2 Introduction

The CIB family of proteins consists of four members termed CIB1- CIB4. Of these, CIB1 is the most widely studied, with multiple binding partners identified, and numerous roles in signal transduction hypothesized (reviewed in Yamniuk and Vogel, 2006). CIB1 was first identified through its association with the platelet-specific integrin αIIb, and numerous studies have since examined its role in platelet activation through regulating the signalling of this integrin (Yamniuk and Vogel, 2006). However, CIB1 has been shown to bind a plethora of other signalling molecules including SK1 (Chapter3), PAK1 (Leisner et al., 2005), Pax3 (Hollenbach et al., 2002), caspase-2s (Ito et al., 2000), presinilin 2 (Stabler et al., 1999; Zhu et al., 2004; Blazejczyk et al., 2006), and DNA-PK (Wu and Lieber, 1997), with predicted roles in apoptosis, proliferation and the DNA damage response (reviewed in Yamniuk and Vogel, 2006). Although few studies have examined the biological function of CIB2, recent studies have confirmed it to be a Ca^{2+} binding protein (Hager et al., 2008), and shown an *in vitro* interaction of CIB2 with both an αIIb cytoplasmic domain peptide (Denofrio et al., 2008), the skeletal muscle integrin α7Bβ1D and SK1 (Chapter 4).

The two remaining CIB family members, CIB3 and CIB4 have been very poorly studied. These proteins share 62% and 64% amino acid sequence similarity with CIB1 respectively (Figure 1.10) (Gentry et al., 2005). Based on sequence alignment, it is anticipated that CIB3 and CIB4 share the putative four EF-hand Ca^{2+} binding domains (EF1-4) that are also present in CIB1. While only EF3 and EF4 of CIB1 are functional in binding Ca^{2+}, it appears likely that CIB3 may also bind an extra Ca^{2+} in EF2 due to the presence of more acidic residues in this loop region compared to CIB1 (Gentry et al., 2005). The large insertion that precludes Ca^{2+} binding in EF1 of CIB1 also appears to be conserved with CIB3 and CIB4, strongly suggesting this EF hand can not bind Ca^{2+} in these proteins (Gentry et al., 2005). In addition, it appears the overall structure of the hydrophobic binding pocket also remains conserved between all of the CIB proteins (Gentry et al., 2005; Denofrio et al., 2008).

Apart from predictions from the sequence analysis, almost no studies have examined the cellular functions of CIB3 or CIB4. In the only study examining CIB3, its mRNA was found to be up-regulated in platelets of CIB1 knock-out mice (Denofrio et al., 2008). Despite a role for CIB1 in regulating signalling through the platelet-specific integrin $\alpha IIb\beta_3$

(Yuan et al., 2006b; Tsuboi, 2002), no defect was observed in activation or aggregation of the CIB1 deficient platelets (Denofrio et al., 2008). Furthermore, recombinant CIB3 was found to interact with an αIIb cytoplasmic tail peptide *in vitro* (Denofrio et al., 2008). Hence, these observations suggest compensation may occur between CIB1 and CIB3 and that both have a similar role in platelet activation. There have been no studies to date on CIB4.

I have previously described the opposing roles of CIB1 and CIB2 in regulating the translocation of SK1 to the plasma membrane (Chapters 3 and 4). While CIB1 is critical in facilitating the agonist-induced translocation of SK1 to the membrane, CIB2 acts as an inhibitor of this process. Thus, CIB1 and CIB2 appear important regulators of SK1-mediated cell signalling and subsequent oncogenesis. Here I have examined the interaction and regulation of SK1 by CIB3 and CIB4. I show that both of these proteins interact with SK1 *in vitro* in a manner recapitulative of CIB2, however the biological outcomes of their interactions with SK1 appear to be different to both of the previously characterized CIB proteins. CIB3 does not appear to contribute to the anti-apoptotic nor oncogenic signalling of SK1 and is largely nuclear localised, leading to questions of whether a physiological role for CIB3 in SK1 signalling is likely. In contrast, CIB4 does appear to contribute to the anti-apoptotic signalling of SK1, yet induces neoplastic transformation of cells in an SK-independent manner, suggesting it may have alternate roles in cellular signalling events not related to SK1.

5.3 Results and Discussion

5.3.1 CIB3 and CIB4 Interact with SK1

To test the interaction between SK1 and CIB3 or CIB4 in mammalian cells, co-immunoprecipitation experiments were performed using lysates from HEK293T cells expressing FLAG-tagged SK1 and HA-tagged CIB3 or CIB4. For CIB4, the presence of SK1 in the anti-HA (CIB4) immunocomplexes supported the existence of an interaction between these two proteins (Figure 5.1A). Unfortunately, CIB3 expressed very poorly in HEK293T cells, and so its interaction with SK1 could not be tested in this manner. To further examine the interactions of CIB3 and CIB4 with SK1, bacterial expression

constructs encoding glutathione *s*-transferase (GST)-CIB3 and GST-CIB4 were generated. *In vitro* pull down experiments were then performed using these proteins and lysates from HEK293T cells expressing SK1. Both GST-CIB3 and GST-CIB4 specifically associated with SK1 from these lysates (Figure 5.1B). The ability of GST-CIB3 to pull down SK1 supported an interaction between these two proteins. The interactions of both CIB3 and CIB4 with SK1 were not surprising, due to their relatively high sequence similarity with CIB1 and hypothesized conserved hydrophobic binding pocket (Gentry et al., 2005; Denofrio et al., 2008). Unfortunately, it was not possible to examine endogenous interactions between these proteins, due to the current lack of available CIB3 and CIB4 antibodies.

CIB1 interacts with SK1 in a Ca^{2+} dependent manner, while CIB2 is able to bind to SK1 independent of metal ions (Chapters 3 and 4). To determine the metal ion dependence of the interactions of CIB3 and CIB4 with SK1, *in vitro* pull down experiments were performed using GST-CIB3, GST-CIB4 and lysates from HEK293T cells expressing SK1 the presence of Ca^{2+}, Mg^{2+} or EGTA. Like CIB2, these CIB family members were able to interact with SK1 in all conditions tested, suggesting their interactions to be independent of metal ions (Figure 5.2). Hence, like CIB2, it appears that the regulation of these CIB-SK1 interactions is different to that of CIB1, which is Ca^{2+} dependent. This could be due to structural variations between CIB1 and its other family members or the use of different binding sites on the CIB proteins. Future elucidation of the structures of CIB2, -3 and -4 may reveal the structural basis for these differences in the metal ion dependence of their interactions with SK1.

5.3.2 CIB3 and CIB4 Interact with both Phosphorylated and Non-Phosphorylated SK1

SK1 is phosphorylated at Ser225, with this phosphorylation an important regulator of SK1 activity, and critical in SK1-associated oncogenic signalling (Pitson et al., 2005). However, CIB1 and CIB2 interact with SK1 independent of the phosphorylation state of this enzyme (Chapters 3 and 4). To determine whether this property is shared with CIB3 and CIB4, I performed pull-down analyses using GST-CIB3 or GST-CIB4 and lysates from HEK293T cells expressing either wildtype SK1 or $SK1^{S225A}$. Both GST-CIB3 and GST-CIB4 were able to bind both wildtype SK1 which is substantially phosphorylated, and the non-

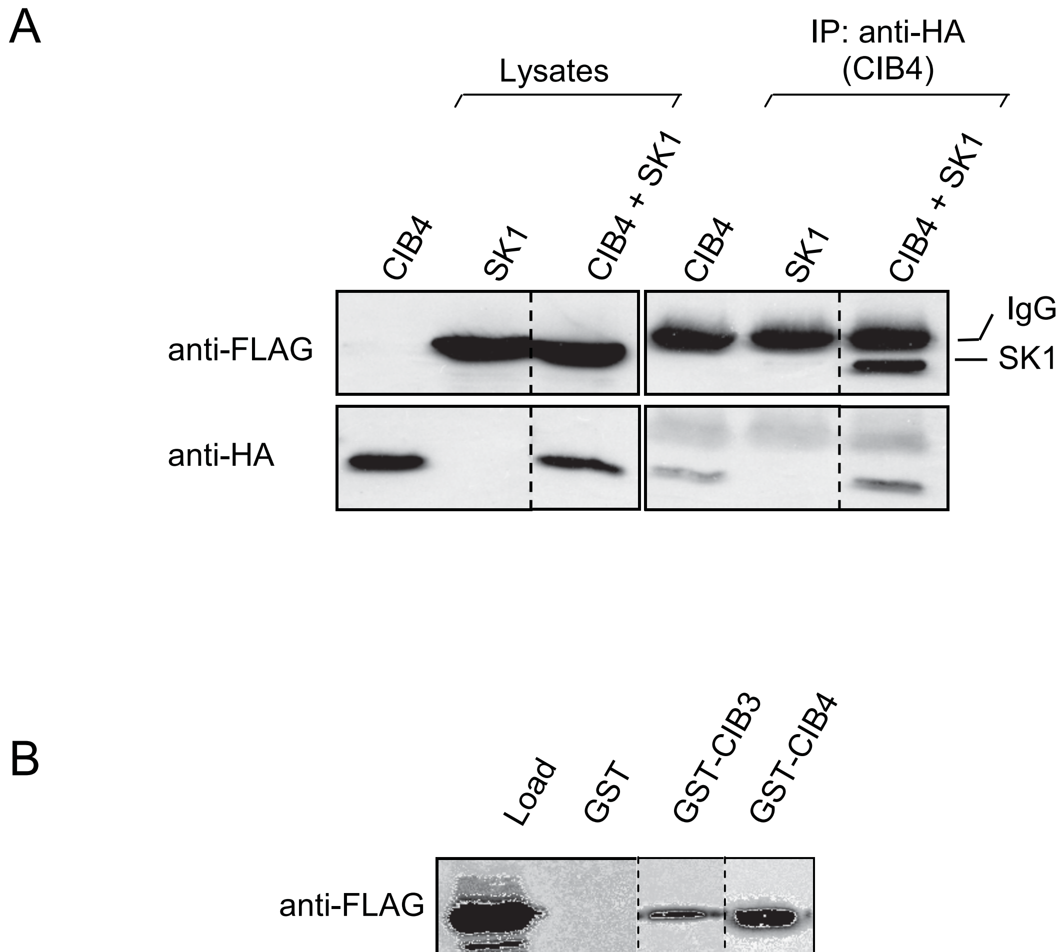


Figure 5.1. CIB3 and CIB4 interact with SK1

(A) SK1 association with CIB4 was examined by coimmunoprecipitation using lysates from HEK293T cells expressing C-terminally HA-tagged CIB4 and FLAG-tagged SK1 either individually or together. Expression of these constructs was confirmed via Western Blot (lysates). CIB4 was immunoprecitated via its HA tag using anti-HA antibodies and associated SK1 detected by Western blot using anti-FLAG antibodies. SK1 was only detected on the immunoprecipitate from the co-expressing cells. IgG is the heavy chain of the anti-HA antibody used in the immunoprecipitation. Data are representative of 3 experiments.

(B) The association of CIB3 and CIB4 with SK1 was further examined using GST-CIB3 and GST-CIB4 to bind SK1 from HEK293T cells expressing this protein (load). Binding specificity was confirmed by the absence of any SK1 bound to GST alone. Data are representative of 4 experiments.

Dividing lines indicate where lanes from the same Western blot have been spliced together to simplify viewing.

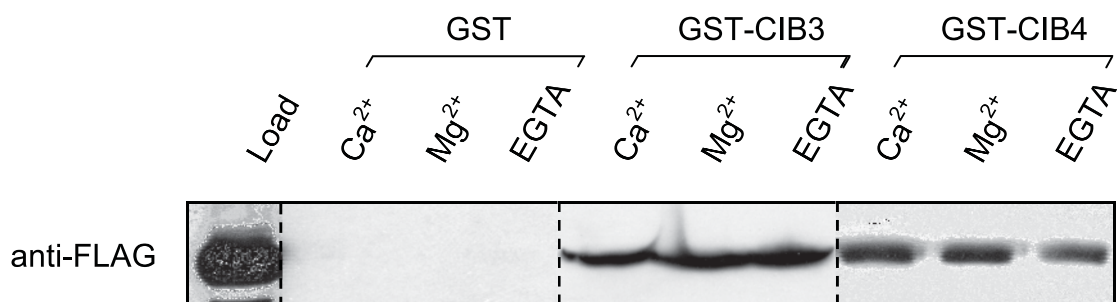


Figure 5.2. CIB3 and CIB4 interact with SK1 independent of metal ions
The metal ion dependence of the CIB3- and CIB4-SK1 interaction was examined using GST-CIB3 or GST-CIB4 to bind SK1 from HEK293T cells expressing this protein (load), in the presence of 2mM $CaCl_2$, $MgCl_2$ or EGTA. Binding specificity was confirmed by the absence of any SK1 bound to GST alone. Dividing lines indicate where lanes from the same Western blot have been spliced together to simplify viewing. Data are representative of 4 experiments.

phosphorylated $SK1^{S225A}$ (Figure 5.3A). Hence, the phosphorylation of SK1 does not appear to regulate its interactions with any of the CIB family members.

5.3.3 CIB3 and CIB4 Interact in the CaM/ CIB1 Binding Site of SK1

As described previously, both CIB1 and CIB2 interact with SK1 in the previously defined CaM binding site (Chapters 3 and 4), with this site critical for the translocation of SK1 to the plasma membrane (Sutherland et al., 2006). To determine whether CIB3 and CIB4 also bind to SK1 in this site, further pull-down analyses were performed using GST-CIB3 or GST-CIB4 and lysates from HEK293T cells expressing either wildtype SK1 or a SK1 variant unable to bind CaM or CIB1/ 2 ($SK1^{F197A/L198Q}$). Again, I found that this site is important for interactions of both CIB3 and CIB4 with SK1, as mutation of this region blocked the association of these proteins (Figure 5.3B). Similar to CIB2, however, there was some evidence of residual binding between the mutated SK1 and CIB3 and CIB4, suggesting that these CIB1 proteins may interact with SK1 in a somewhat different manner to CIB1. However, as each of the CIB proteins appear to utilise at least some of the same residues in SK1 for interaction, it is likely that all four CIB proteins compete for binding to SK1.

5.3.4. CIB3 and CIB4 are Myristoylated

Both CIB1 and CIB2 are myristoylated proteins, with CIB1 utilizing its fatty acid moiety to exhibit Ca^{2+}-myristoyl switch behaviour. To determine whether CIB3 and CIB4 are also myristoylated, HeLa cells were transiently transfected with CIB3 or CIB4 and metabolically labelled with ^{3}H-myristic acid. Fluorography performed on the CIB proteins immunoprecipitated from the cell lysates demonstrated that both CIB3 and CIB4 are myristoylated (Figure 5.4). Myristoylation of CIB3 was anticipated due to its *N*-terminal amino acid sequence conforming to the general myristoylation consensus of ^{1}M-G-X-X-X-S/T (Utsumi et al., 2004). Although CIB4 also has a Glycine at position 2, it deviates from this consensus with an Arginine at amino acid position 6 (^{1}M-G-Q-C-L-R). Although several variations from this consensus rule do exist, none of them include a prediction for myristoylation of a sequence consistent with that of CIB4. In addition, Utsumi and

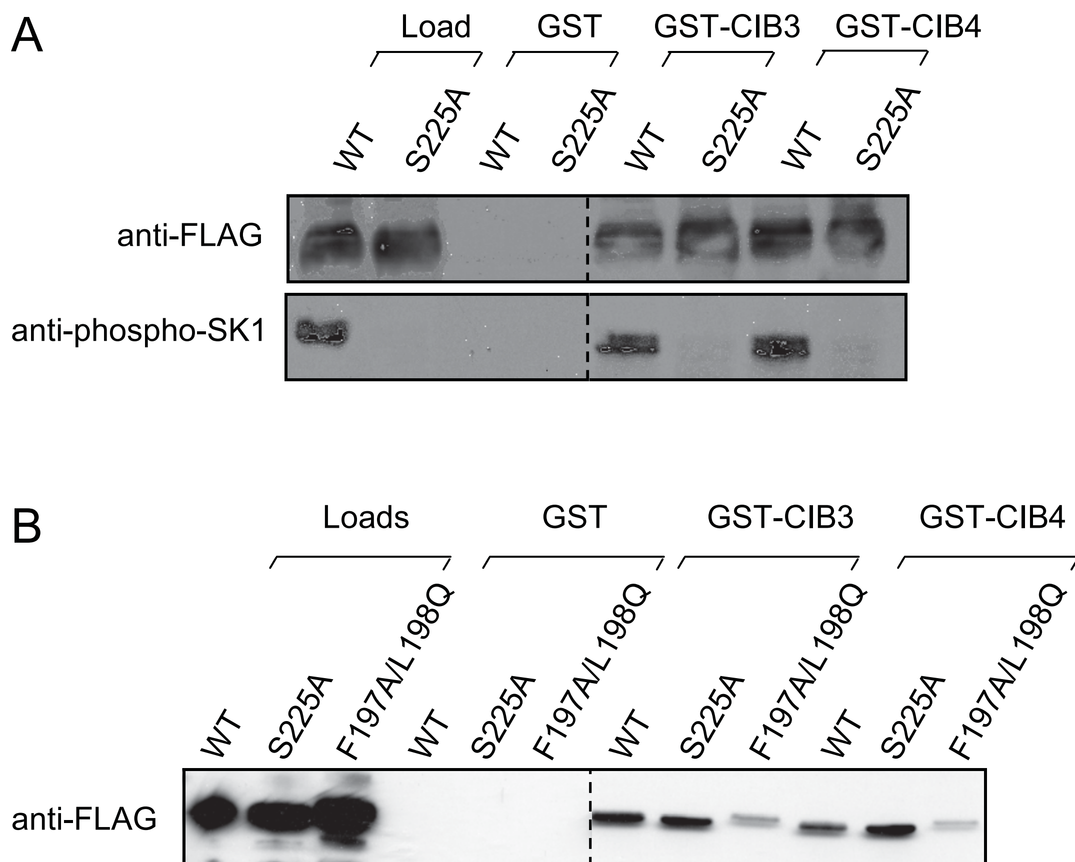


Figure 5.3. Further characterisation of the CIB3- and CIB4-SK1 interactions.

(A) The ability of CIB3 and CIB4 to bind both phosphorylated and non-phosphorylated SK1 was tested using recombinant GST-CIB3 and GST-CIB4 and lysates from HEK293T cells expressing either wildtype SK1 (WT) or $SK1^{S225A}$ (S225A). Total SK1 was detected using anti-FLAG antibodies, while phospho-SK1 was detected using anti-phospho-SK1 antibodies. Binding specificity was confirmed by the absence of any SK1 bound to GST alone.

(B) The ability of CIB3 and CIB4 to bind the 'CaM' binding site mutant of SK1 (F197A/L198Q) in comparison to either wildtype SK1 (WT) or non-phosphorylated SK1 (S225A) was tested using recombinant GST-CIB3 and GST-CIB4 and lysates from HEK293T cells expressing either wildtype SK1 (WT), $SK1^{S225A}$ or $SK1^{F197A/L198Q}$. SK1 variants were detected using anti-FLAG antibodies. Binding specificity was confirmed by the absence of any SK1 bound to GST alone.

Data are indicative of 3 experiments.

Dividing lines indicate where lanes from the same Western blot have been spliced together to simplify viewing.

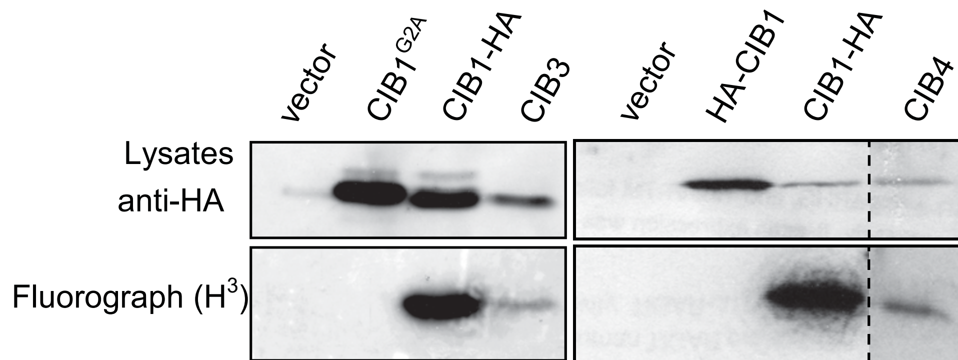


Figure 5.4. CIB3 and CIB4 are myristoylated

The myristoylation status of CIB3 and CIB4 were determined by metabolically labelling HEK293T cells expressing C-terminally HA-tagged CIB3 or CIB4 with ^{3}H myristic acid. N-terminally HA-tagged CIB1 (HA-CIB1) or C-terminally HA-tagged CIB1^{G2A} were used as a negative controls, while C-terminly HA-tagged CIB1 (CIB1-HA) was the positive control. CIB proteins were immunoprecipitated from these cell extracts using anti-HA antibodies, proteins separated by SDS-PAGE and the signal from the incorporated ^{3}H detected by fluorography. Dividing lines indicate where lanes from the same Western blot have been spliced together to simplify viewing. Results are representative of at least 2 experiments.

colleagues examined the sequence of 78 known myristoylated proteins, and none of these were found to have an arginine at position 6, as CIB4 does (Utsumi et al., 2004). Furthermore, using an *in vitro* translation model whereby the first nine amino acids of an *N*-terminal consensus motif upstream of TNF were systematically mutated to determine their importance in myristoylation, the placement of an Arginine at position 6 did not result in myristoylation of this protein (Utsumi et al., 2004). Hence, the finding that CIB4 is myristoylated is quite unexpected.

5.3.5. CIB3 and CIB4 do not appear to Act Like Ca^{2+}-Myristoyl Switches

Ca^{2+}-myristoyl switch proteins typically translocate to membranous regions of the cell upon Ca^{2+} flux, enabled by exposure of their fatty acid moiety into the cellular environment (Meyer and York, 1999). This behaviour is observed with CIB1 but not CIB2 upon treatment with the Ca^{2+} ionophore, ionomycin, or upon stimulation with phorbol ester (PMA), a known SK1 activator (Johnson et al., 2002; Pitson et al., 2005). To examine the cellular distribution of CIB3 or CIB4 and their ability to act like Ca^{2+}-myristoyl switches, I examined their localisation in HeLa cells both before and after PMA or ionomycin stimulation. Prior to stimulation, the localisation of CIB4 appeared very similar that of both CIB1 and CIB2, with all three of these proteins largely cytoplasmic (Figure 5.5). Upon either PMA or ionomycin addition, this localisation of CIB4 did not appear to change, remaining cytoplasmic, very much emulative of the behaviour of CIB2 (Figure 5.5).

The localisation of CIB3 was quite different to that observed with any of the other CIB proteins. Under all conditions tested, CIB3 appeared to be principally expressed in large discrete structures in the nucleus (Figure 5.5). These differed in size, shape and number dependent upon the particular cell viewed. They did not align with nucleolar structures, as in several instances, these were also visible in the nucleus and absent of CIB3 staining. As with any study of this nature, it is possible that the overexpression of this protein has disrupted its physiological localisation, however, as no CIB3 antibodies are currently available, it was not possible to overcome this hurdle at this time. Following PMA stimulation, CIB3 localisation appeared unchanged in the majority of cells. However, in a small number of CIB3 expressing cells, some CIB3 was visible at the plasma membrane following PMA stimulation. This may represent a small cytoplasmic fraction of CIB3

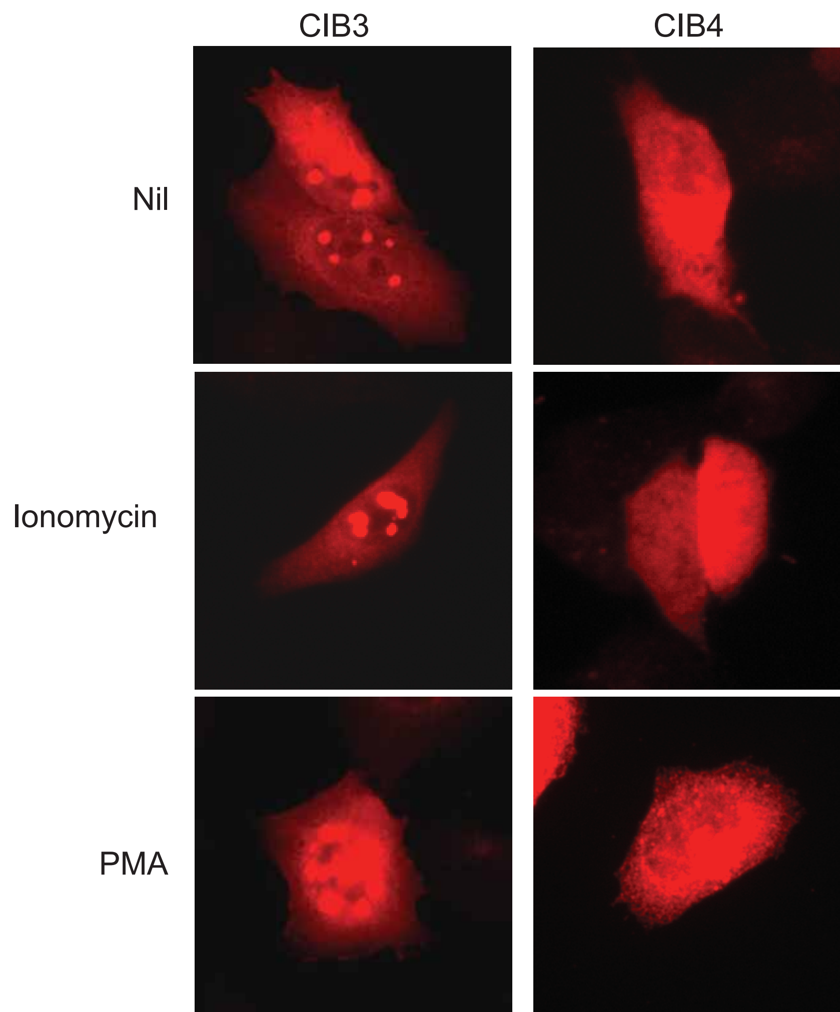


Figure 5.5. CIB3 and CIB4 do not act like Ca^{2+}-myristoyl switches

Fluorescence microscopy of HeLa cells expressing either C-terminally HA-tagged CIB3 or CIB4 detected by anti-HA antibodies following stimulation with 2 µM ionomycin for 2 mins, or 1 µg/ml PMA for 30 mins. Results are representative of over 100 cells examined in 3 experiments.

translocating to the membrane upon stimulation. However, as this re-localisation of CIB3 was not apparent upon ionomycin stimulation, this phenomenon does not appear to be Ca^{2+} mobilization-dependent, and hence CIB3 is not likely to function as a Ca^{2+}-myristoyl switch.

Overall, it was quite apparent that like CIB2, CIB4 does not act as a Ca^{2+}-myristoyl switch, and as such it is unlikely to have a role in mediating the translocation of SK1 to the plasma membrane. Although the localisation of CIB3 appeared slightly ambiguous, due to the main bulk of CIB3 residing within the nucleus, it is also unlikely to regulate the translocation of cytoplasmic SK1 in these cells.

5.3.6 Expression of CIB4 Inhibits Agonist-Induced Translocation of SK1 to the Plasma Membrane

I have previously shown the ability of CIB2 to act in an opposing manner to CIB1 in inhibiting the agonist-induced translocation of SK1 to the plasma membrane (Chapter 4). As CIB4 conserved both its cellular localisation and its inability to exhibit Ca^{2+} myristoyl switch behaviour with CIB2, I investigated the possibility that it too may block SK1 translocation. Hence, the localisation of endogenous SK1 was examined in cells expressing CIB4, with and without exposure to PMA, a known inducer of SK1 translocation (Johnson et al., 2002; Pitson et al., 2003). Following PMA stimulation, endogenous SK1 showed clear plasma membrane localization in control cells as anticipated, demonstrating the effectiveness of the PMA stimulation. In CIB4 expressing cells, however, no such SK1 relocalisation to the plasma membrane was apparent (Figure 5.6A). These results are very similar to those observed with expression of CIB2, and suggest that CIB4 may also act to inhibit SK1 translocation. Thus it is possible that the inhibition of SK1 translocation may be due to overexpressed CIB4 out-competing endogenous CIB1 for binding to SK1, thus retaining this enzyme in the cytoplasm despite agonist stimulation.

Due to its mainly nuclear expression, a role for CIB3 in the regulation of SK1 localisation seemed unlikely. Nevertheless, the localisation of endogenous SK1 in CIB3 expressing cells was also examined. As anticipated, endogenous SK1 was still able to translocate to the plasma membrane following PMA stimulation (Figure 5.6B). Hence, it appears that unlike

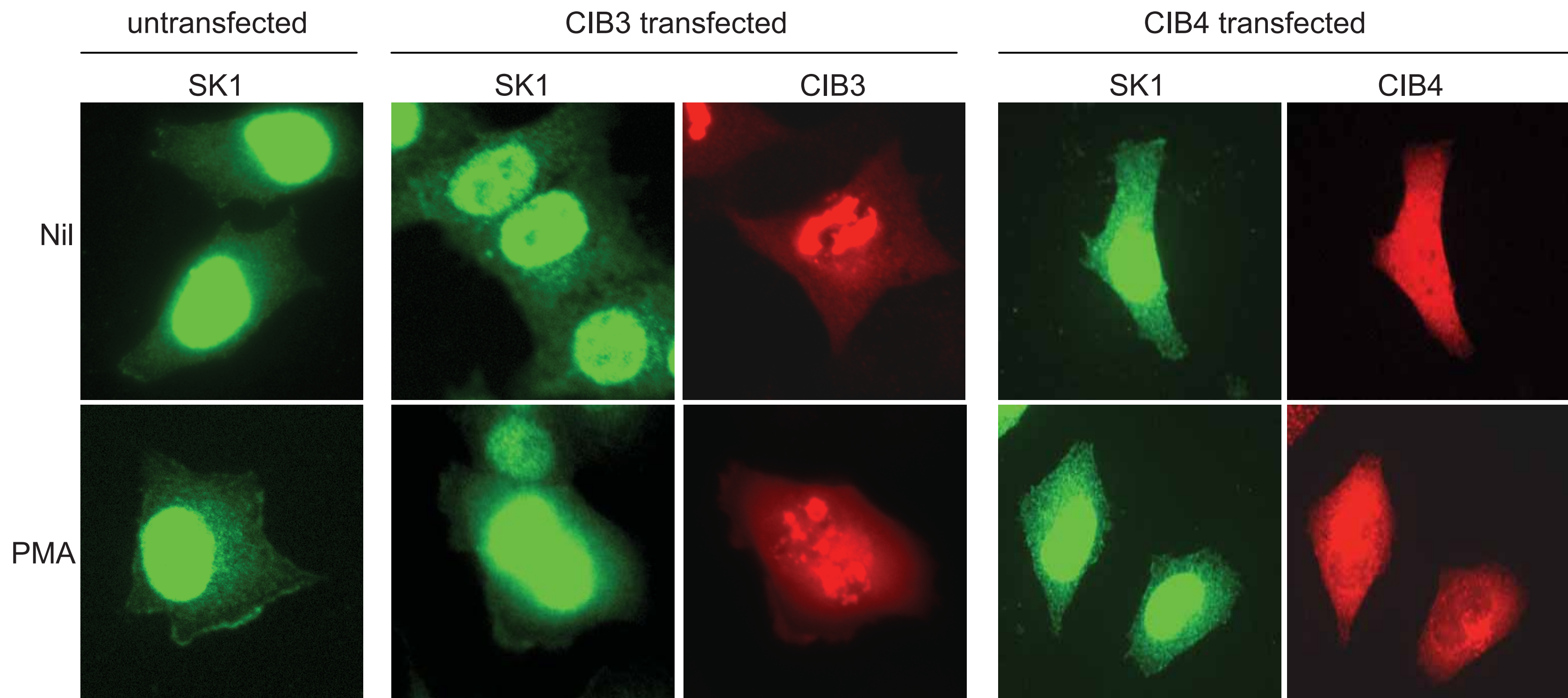


Figure 5.6. Expression of CIB4, but not CIB3 blocks agonist-induced translocation of endogenous SK1
Fluorescence microscopy of either untransfected HeLa cells or cells expressing CIB3 (red) or CIB4 (red) with or without PMA stimulation. Cells expressing CIB3 or CIB4 (red) were identified using its HA tag, while endogenous SK1 (green) was visualized using anti-SK1 antibodies. Images are representative of 100 cells examined in two independent experiments.

CIB2 and CIB4, CIB3 does not have an inhibitory role in the agonist-induced translocation of SK1 to the plasma membrane. This is most likely due to the restricted nuclear localization of CIB3.

Notably, quite strong nuclear staining of endogenous SK1 was visible in the nucleus of all cells examined. This nuclear staining of SK1 did not alter upon agonist stimulation, and hence, it seems likely that only cytoplasmic SK1 that translocates to the plasma membrane. This is in agreement with our previous observations (Chapters 3 and 4), and those of others (Johnson et al., 2002). However, as this nuclear staining of SK1 is quite strong, it is also possible that due to saturation, a decrease in nuclear SK1 occurred but was simply not detected. In addition, SK1 has two functional nuclear export signal sequences, and has been found to accumulate in the nucleus upon inhibition of the nuclear export reporter CRM1 (Inagaki et al., 2003). Hence, a nuclear role for SK1, in addition to its cytoplasmic signalling may exist in some cells under certain conditions. Hence, the nuclear localisation of CIB3 in this respect was quite interesting, and suggests the potential for a CIB3-SK1 interaction in this location. However, as CIB3 expression had no affect on SK1 translocation, this potential CIB3-regulated nuclear function of SK1 is most likely separate from the plasma membrane translocation of this enzyme, and likely has alternate roles in the cell.

5.3.7 Expression of CIB4 Enhances Cell Susceptibility to TNF-α-Induced Apoptosis

As previously described, SK1 activation is critical in the prevention of TNF-α-induced apoptosis (Xia et al., 2002). I have also previously shown the ability of both a dominant-negative CIB1, and CIB2 to enhance cellular susceptibility to TNF-α induced apoptosis, presumably through inhibition of the SK1 signalling pathway (Chapters 3 and 4). As CIB4 expression appeared to emulate that of CIB2 in its ability to block the agonist-induced translocation of endogenous SK1 to the plasma membrane, I examined whether CIB4 could also affect the TNF-α stimulated apoptotic/ anti-apoptotic pathway. To test this, apoptosis was measured in HeLa cells stably expressing CIB3 or CIB4, following treatment with TNF-α and cycloheximide (CHX) for 18 hours. Results showed that, similar to both the dominant negative $CIB1^{G2A}$ and CIB2 (Figure 3.12), cells expressing CIB4 underwent

higher levels of apoptosis following TNF-α and CHX treatment in comparison to the vector cells (Figure 5.7A). Meanwhile, expression of CIB3 did not appear to alter TNF-α induced apoptosis, with comparable results seen between these and vector cells (Figure 5.7A). The increased apoptosis observed with CIB4 was further confirmed by measurement of caspase 3/7 activity in these cells (Figure 5.7B). Hence, these results add further support to the potential of CIB4, like CIB2, to act as an endogenous inhibitor of SK1 translocation. The inability of CIB3 to affect the anti-apoptotic signalling of SK1 was not surprising and adds further support to the suggestion that CIB3 is not involved in the regulation of SK1 translocation to the plasma membrane, and the downstream signalling associated with this event.

5.3.8 CIB4 Transforms Cells in an SK1-Independent Fashion

SK1 activation and subsequent translocation to the plasma membrane are critical for its oncogenesis. I have previously demonstrated the SK-dependent oncogenic nature of CIB1 (Chapter 3), through its role in mediating the translocation of this enzyme. I have also previously demonstrated a role for the CIB1-dependent translocation of SK1 in Ras oncogenesis, with 'dominant-negative' CIB1 or expression of CIB2 able to block Ras transformation (Chapter 3 and Chapter 4, respectively). To investigate the ability of CIB3 and CIB4 to induce neoplastic transformation, I generated NIH3T3 cells stably expressing either of these proteins and examined the ability of the cells to form foci in culture. As expected, CIB3 expression did not induce focus formation (Figure 5.8). This was not surprising considering the apparent lack of a role for CIB3 in the agonist-induced signalling pathway of SK1. Quite surprisingly, however, expression of CIB4 resulted in numerous foci (Figure 5.8). This was quite unexpected due to previous evidence suggesting an inhibitory role for CIB4 in the signalling pathway of SK1. Notably, addition of the SK inhibitor, SK-I, did not affect the formation of these foci (Figure 5.8). Hence, the potential for neoplastic transformation of cells by CIB4 appears to be independent of SK.

To determine whether, like CIB2, CIB3 or CIB4 can affect Ras-induced neoplastic transformation, NIH3T3 cells expressing CIB3 or CIB4 alone or coexpressed with oncogenic H-Ras (V12-Ras) were examined for neoplastic transformation via focus formation assays. Results showed that CIB3 was not able to inhibit Ras transformation

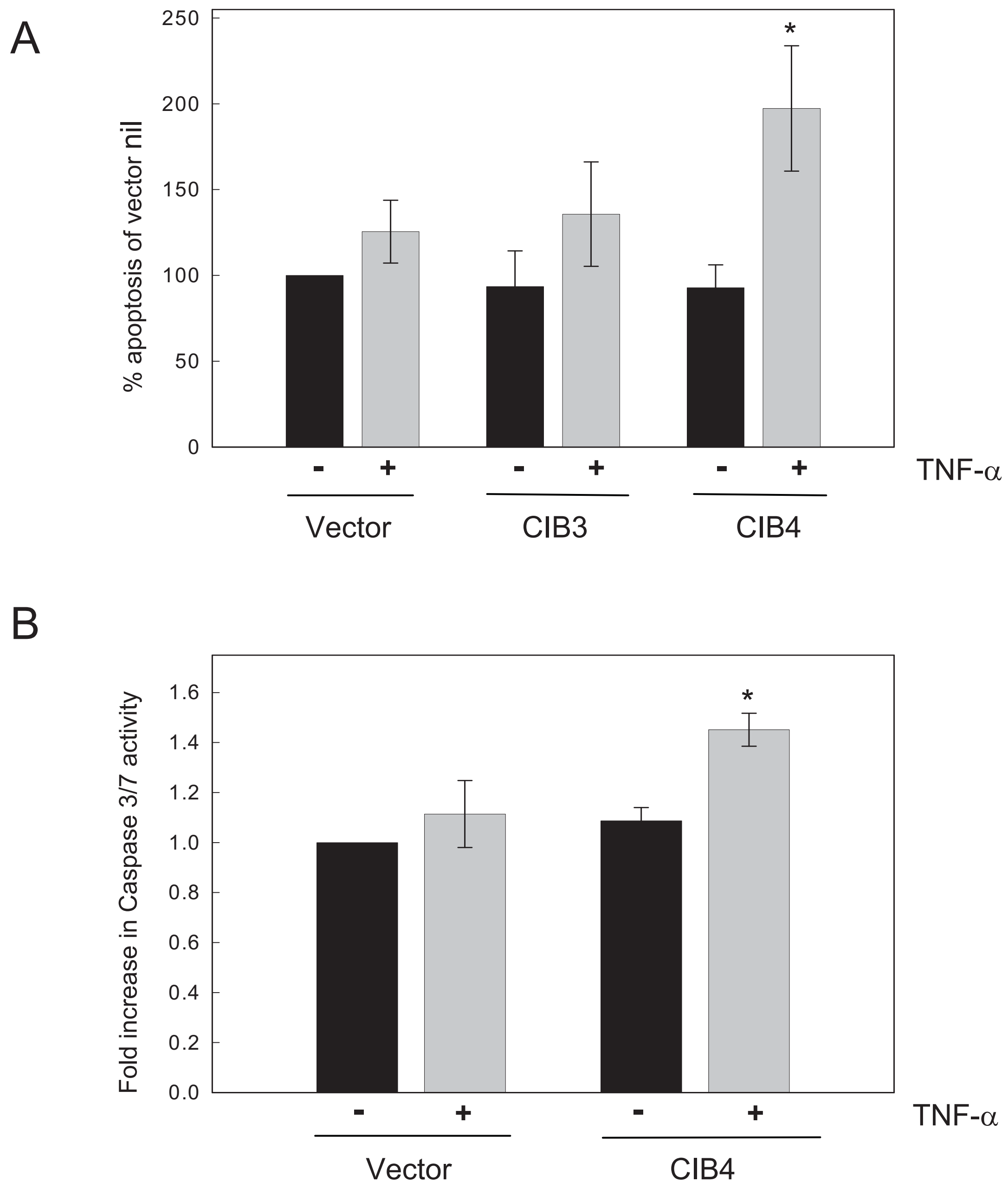


Figure 5.7. CIB4, but not CIB3, enhances cell susceptibility to TNF-α-induced apoptosis
(A) To examine whether CIB3 or CIB4 affect the TNF-α stimulated apoptotic/ anti-apoptotic pathway these proteins were expressed in HeLa cells and apoptosis measured after treatment of the cells with TNFα and CHX for 18 h. Apoptosis was measured through the percentage of floating cells with ~99% of these floating cells showing positive cell surface staining for the apoptosis marker Annexin V. Data are the mean percentage increase ± SD of three independent experiments relative to nil treated vector cells. Statistical significance was calculated by an unpaired t-test. * $p < 0.05$
(B) Caspase 3/7 activity assays were performed on the above cell lysates, with caspase 3/7 activity calculated per cell. Data represents mean increase ± SD of three independent experiments relative to nil treated vector cells. Statistical significance was calculated by an unpaired t-test. * $p < 0.02$

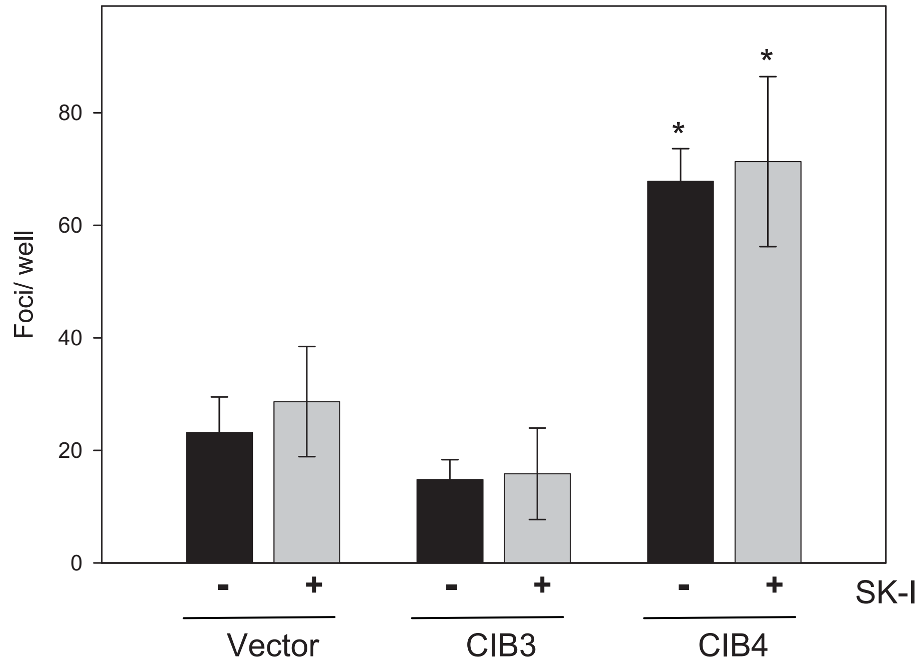


Figure 5.8. CIB4 transforms cells independent of the SK signalling pathway

One day after seeding into 6-well plates, NIH3T3 cells stably expressing CIB3, CIB4 or empty vector were cultured for 3-4 weeks, with media replaced every 2-3 days in the presence or absence of 5 mM SK-I. Foci were scored after fixing with methanol and staining with 0.01% methyl violet. Data is mean ± SD of three independent experiments with statistical significance calculated by an unpaired t-test. * $p < 0.01$

(Figure 5.9). Again, this was not surprising due to the lack of an affect of CIB3 in SK1 signalling. Similarly, CIB4 did not have any inhibitory affect on Ras-induced neoplastic transformation (Figure 5.9). This was expected, considering the observed ability of CIB4 to induce neoplastic transformation described above.

Very little is known regarding potential cellular functions of CIB4. By comparison with CIB1, however, it is likely that CIB4 may bind numerous other proteins in the cell, and contribute to multiple signalling pathways. Hence, the transforming ability of this protein may be due to an as yet identified role in another signalling pathway in the cell. Regardless, the oncogenic potential of CIB4 does not appear to be mediated through SK1. Consequently, the role of CIB4 in respect to the SK1 signalling pathway remains currently undefined. Further studies are required to determine whether other CIB4 interacting proteins exist, and how these function to regulate cellular signalling events.

5.4 Conclusions

I have previously described the two CIB homologues CIB1 and CIB2 and their very divergent roles in the regulation of SK1 signalling through their influence on the translocation of this enzyme to the plasma membrane (Chapters 3 and 4). While CIB1 is critical for the agonist-induced translocation of SK1 to the plasma membrane, integral for its pro-survival, pro-proliferative and oncogenic signalling, CIB2 acts in an inhibitory manner to block this event. In this study, I have characterized two other proteins of the CIB family, CIB3 and CIB4, and their interactions with SK1. Similar to CIB2, both CIB3 and CIB4 associated with SK1 independent of Ca^{2+} or SK1 phosphorylation state, suggesting basal interactions of these proteins with SK1.

The identification that all CIB protein family members utilise the same residues on SK1 for interaction suggests that competition between these proteins for SK1 binding is likely. Further studies characterizing the binding affinities of each of these proteins for SK1 will most likely shed further light on the regulation of the SK1-CIB protein interactions. It is likely that the interactions of each of the CIB family members with SK1 are also regulated by their relative expression. Although CIB1 and CIB2 appear to be ubiquitously expressed in a variety of human tissues (Seki et al., 1999; Shock et al., 1999), CIB3 and CIB4 are

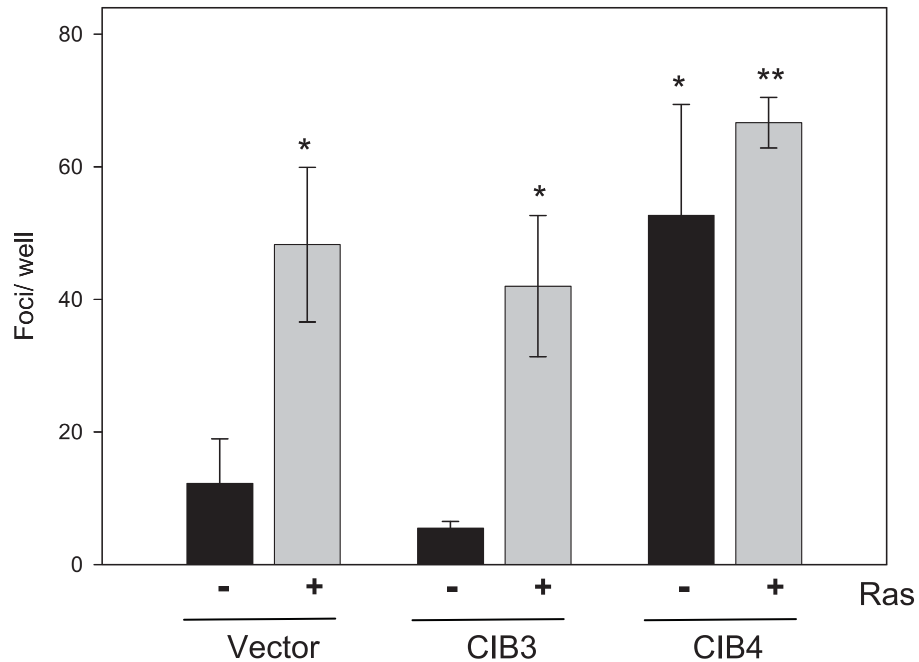


Figure 5.9. Neither CIB3 nor CIB4 affect the Ras oncogenic pathway

One day after seeding into 6-well plates, NIH3T3 cells stably expressing CIB3, CIB4 or empty vector were transfected with V12-Ras and cultured for 3-4 weeks, with media replaced every 2-3 days. Foci were scored after fixing with methanol and staining with 0.01% methyl violet. Data is mean ± SD of three independent experiments with statistical significance calculated by an unpaired t-test. * $p < 0.02$, ** $p < 0.0002$

predicted to have a more selective expression profile. From a search of Expressed Sequence Tags (ESTs) in the NCBI database, dbEST, CIB3 expression appears restricted to adults in the connective tissue, and has been identified in muscle tissue tumour. Meanwhile, in this same database, CIB4 is predicted to be expressed in ascites, kidney, prostate and testis tissues, and has been identified in gastrointestinal and germ cell tumours. A more thorough Northern Blot analysis of the CIB proteins expression in human tissues may provide further insight into whether these interactions with SK1 are regulated in a tissue specific manner.

Despite both CIB3 and CIB4 being myristoylated, neither acted as Ca^{2+}-myristoyl switches. The myristoylation of CIB4 is a novel finding, with its *N*-terminal sequence not previously identified as being modified by addition of a myristoyl moiety. As neither CIB3 nor CIB4 appeared to be membrane localised, the function of their myristoylations remains to be determined. It is also likely that the myristoyl moieties of these proteins are hidden or sequestered into their structures, to enable their respective localisations.

CIB3 appeared to be largely localized to discrete regions within the nucleus, and it did not act like a Ca^{2+}-myristoyl switch. Hence, a role for CIB3 in mediating the translocation of cytoplasmic SK1 is unlikely. This is supported by the lack of any observed effects of CIB3 expression on SK1 translocation, or anti-apoptotic signalling upon agonist stimulation. As CIB3 expression appears to be restricted to the nucleus, and it is likely to be cytoplasmic SK1 that translocates to the plasma membrane upon agonist stimulation resulting in pro-survival and pro-proliferative signalling, it is difficult to perceive a role for CIB3 in the regulation of SK1 signalling in this context. Of note, however, quite strong endogenous SK1 staining was visible in the nucleus of the cells examined in this study. Hence, a nuclear role for SK1, in addition to its cytoplasmic signalling may exist in some cells under certain conditions. Due to the mainly nuclear localization of CIB3, it is tempting to speculate a CIB3-regulated role for SK1 in the nucleus.

The ability of CIB4 to block endogenous SK1 translocation in addition to increasing cellular susceptibility to TNF-α induced cell death, presumably through the impediment of SK1 signalling, showed promising signs that like CIB2, CIB4 may also act as an endogenous inhibitor of SK1 translocation. However, the ability of CIB4 to induce neoplastic transformation in an SK-independent manner suggests that it likely has additional binding partners and signalling roles in the cell, masking my ability to determine the biological

function of the CIB4-SK1 interaction. Further studies are also needed to determine the ability of CIB4 to lead to tumour formation in mice, with these experiments necessary to confirm the true oncogenic nature of CIB4.

Overall, this study has identified two potential new binding partners for SK1. The possibility for a CIB3-regulated nuclear role for SK1 is quite interesting, with further studies required to determine if a true interaction between CIB3 and SK1 in the nucleus exists, and what the biological function of this may be. Our current understanding of the role of CIB4 in SK1 signalling remains incomplete, with the potential for multiple binding partners for CIB4 in the cell. Further studies should examine the full compliment of CIB1-4 binding partners in the cell, in an attempt to determine the reasons for such varied signalling responses between these four closely related proteins. These studies may also shed some light onto the origin of the potential oncogenic nature of CIB4.

Chapter 6: Sphingosine Kinase is Critical in Collagen-Induced Platelet Aggregation

6.1 Abstract

Platelet activation at the site of vascular injury is mediated through a number of signalling events initiated by exposure to soluble, activating agents. This initiates inside-out signalling resulting in platelet shape change and the binding of fibrinogen, ultimately resulting in platelet aggregation. A number of agonists are capable of stimulating platelet activation, including collagen, thrombin and S1P. Although S1P is already present in the plasma, activated platelets further release this phospholipid, which is then thought to activate neighbouring platelets, in a feed-forward manner. Although a role for S1P in platelet aggregation has been established, there is limited and conflicting evidence describing the function of SK1 in this process. While altered SK1 activity upon platelet activation is not evident, an increase of SK1 protein concentration at the plasma membrane has been observed. CIB1, the protein responsible for mediating SK1 translocation in other mammalian cells, is expressed in platelets, regulating the activity of the integrin αIIbβ3. A potential link between CIB1 and SK1 recruitment to the plasma membrane upon platelet activation is an intriguing possibility yet to be examined. In addition, the S1P receptors responsible for conveying the S1P-induced platelet activation have not completely been elucidated. In this study, I investigated the affects of the two SK inhibitors DMS and SK-I in addition to the $S1P_2$ receptor antagonist JTE-013 on platelet aggregation and fibrinogen binding in response to a number of different agonists. I show that both SK-I and JTE-013 specifically inhibit the collagen-induced platelet aggregation, with neither of these inhibitors affecting either shape change or fibrinogen binding. Hence it is likely that the involvement SK1 and $S1P_2$ in collagen-mediated platelet aggregation occur subsequent to these events. Neither SK-I nor JTE-013 affected platelet aggregation upon ADP stimulation, however in contrast, SK-I considerably reduced fibrinogen binding upon stimulation with this agonist. As these results were conflicting, further experimentation is required to clarify the role of SK1 in ADP-induced platelet activation.

6.2 Introduction

Platelets function at the site of vascular injury to prevent further blood loss and re-instate vascular integrity. Soluble secreted agonists at the site of the wound activate these anucleate blood cells, enabling them to bind extracellular matrix proteins such as fibrinogen. This enables the platelets to link together in aggregates, forming a blood clot.

Platelet aggregation is mediated by several signalling events. Initial platelet activation results in inside-out signalling, where the integrin, αIIbβ3 receives intracellular signals, causing it to undergo a rapid conformational change. This integrin, now in a high affinity binding state, can then bind soluble ligands, such as fibrinogen, initiating further intracellular signalling in the platelet (outside-in signalling), required for platelet spreading and clot retraction (reviewed in Yamniuk and Vogel, 2006).

In addition to factors such as thrombin, collagen and ADP, S1P can also result in platelet activation. Plasma S1P is a result of its secretion from numerous cell types with both red blood cells and platelets themselves believed to be the largest contributors (reviewed in Yatomi, 2008), along with a diverse range of other cells, including the vascular endothelium (Venkataraman et al., 2008). Platelets store abundant levels of S1P due to their high SK activity, yet lack of S1P lyase (Buehrer and Bell, 1992; Stoffel et al., 1973a; Stoffel et al., 1973b; Yatomi et al., 1997). Consequently, S1P accumulates in these cells, to be released into the plasma upon stimulation (Yatomi et al., 1997). Once in the extracellular environment, it is believed that S1P can act in a paracrine manner, activating neighbouring platelets and resulting in platelet aggregation and clot formation (Yatomi et al., 1995a). The addition of exogenous S1P to human platelets also mobilises intracellular Ca^{2+}, with the dose response for Ca^{2+} release closely correlated with that required for shape change (Yatomi et al., 1995a). Hence S1P is suggested to be an paracrine stimulator of platelet activation and has a likely role in platelet-mediated wound repair (Yatomi, 2008).

Until very recently, neither the S1P receptors expressed on platelets nor the intracellular signalling mechanism underlying effects of S1P in platelets had been clearly characterised. Randriamboavonjy et al. (2009) have begun to elucidate the mechanism of the S1P-induced platelet activation, identifying expression of the S1P receptor, $S1P_2$ on human platelets, while $S1P_1$ and $S1P_3$ expression were not detected. The use of an $S1P_2$ antagonist in this

study inhibited the S1P-induced platelet aggregation, and modestly attenuated the S1P-induced increase in intracellular Ca^{2+}. In contrast, while the use of an $S1P_{1/3}$ antagonist also inhibited the S1P-induced platelet aggregation, it had no affect on intracellular Ca^{2+} (Randriamboavonjy et al., 2009). This suggests $S1P_1$ and $S1P_3$ may also be present on the platelet surface below detectable limits, contributing to different platelet responses to $S1P_2$. S1P treatment of human platelets also resulted in an activation and translocation of RhoA from the cytosol to the plasma membrane, with this event inhibited by both $S1P_2$ and $S1P_{1/3}$ antagonism. As $S1P_2$ was the main receptor that appeared to be expressed on human platelets, these authors hypothesised this receptor to be the main mediator of S1P-induced platelet activation through a cascade involving G_q and PLCβ1 (Randriamboavonjy et al., 2009).

Despite the role of S1P in platelet activation being quite well studied, the precise function of SK1 in this process remains to be determined. This may be due to a unique action of SK1 in blood platelets compared to other well studied systems. In most cells, the presence of S1P lyase in addition to a low basal SK1 activity results in low cellular S1P levels. The activation of SK1 is required to generate a greater pool of S1P to mediate further downstream signalling. In platelets, however, SK1 basal activity is high, and S1P lyase is absent, generating a large supply of S1P (Buehrer and Bell, 1992; Stoffel et al., 1973a; Stoffel et al., 1973b). Additionally, no overall change in SK1 activity has been observed upon activation with a wide range of agonists, despite these stimulators strongly inducing platelet activation (Yatomi et al., 1997; Ohmori et al., 2005). Hence, it has been unclear whether SK1 plays a role in platelet activation.

Despite no overall change in SK1 activity being observed upon platelet activation, Yatomi and colleagues (1996) were able to demonstrate a requirement for SK1 in TRAP (thrombin receptor-activating protein) -induced platelet activation, with the SK1 inhibitor, *N,N*-dimethylsphingosine (DMS), preventing platelet aggregation. In separate studies, Ca^{2+} mobilisation specifically induced by collagen or convulxin (an agonist of the collagen receptor, GPVI) was inhibited by the use of DMS in platelets, but this was not seen with G-protein stimulating agonists such as ADP, TRAP or thrombin (Ohmori et al., 2005; Yang et al., 1999).

It has been suggested that SK1 may influence platelet activation through a change in localisation of this enzyme, rather than an increase in overall activity. Ohmori and colleagues (2005) were able to demonstrate an increase in SK1 activity in the membrane fraction of platelets when specifically activated through the collagen receptor. Hence, they postulated that activation of the collagen receptor may result in an increase in S1P concentration close to the plasma membrane within platelets. Interestingly, CIB1, the protein I have shown responsible for mediating SK1 translocation in other mammalian cells (Chapter 3) is expressed in platelets and has a postulated role in platelet activation through regulating the activity of the integrin αIIbβ3. CIB1 has been demonstrated to preferentially bind activated αIIbβ3, with this event necessary for the release of ADP from platelet granules (Naik and Naik, 2003a). Furthermore, CIB1 has been shown to recruit Wiskott-Aldrich Syndrome Protein (WASP) to αIIbβ3 following agonist stimulation, and enhance platelet adhesion, probably through concentration of the actin polymerizing activity of WASP near αIIbβ3 (Tsuboi et al., 2006). Whether a link between CIB1 and SK1 recruitment to the plasma membrane exists in platelet activation is yet to be examined.

Although it appears that SK1 activity may be specifically involved in collagen-induced platelet activation, a limiting factor in these studies was the sole use of DMS as an SK inhibitor. In addition to inhibiting SK1, DMS also has effects on other signalling proteins (Woodcock, 2006), including the inhibition of PKC activity (Igarashi et al., 1989), and enhancement of PKC activity (Ma et al., 2005). In addition, none of the above studies determined the stage of platelet activation at which SK1 is critical. Hence, in an attempt to clarify a specific role of SK1 in platelet activation, I have utilised the specific SK inhibitor SK-I (French et al., 2003) and investigated these inhibitor effects on collagen and ADP dependent platelet aggregation, as well as ADP, CRP (collagen related peptide) and TRAP-induced fibrinogen binding. Furthermore, I have also utilised the $S1P_2$ receptor antagonist JTE-013 in this system to determine whether this S1P receptor has a role in platelet activation induced by these agonists. Here I demonstrate a specific role for both SK1 and $S1P_2$ in collagen-mediated platelet aggregation.

6.3 Specific Materials and Methods

6.3.1 Preparation of Platelets from Human Whole Blood

6.3.1a Buffers

7 x Acid Citrate Dextrose containing theophylline (ACD)

Sodium Citrate	85mM
Citric Acid	72.9mM
D-Glucose	110mM
Theophylline	70mM

10x Platelet Wash Buffer (PWB)

K_2HPO_4	43mM
Na_2HPO_4	43mM
NaH_2PO_4	243mM
NaCl	1.13M
D-Glucose	55mM
Theophylline	100mM

1x PWB, pH 6.5, conductivity 13-15

	Per 50mls
10x PWB	5mls
10% BSA	2.5mls
dH_2O	42.5mls

10x Tyrode's Buffer

$NaHCO_3$	120mM
Hepes	100mM
NaCl	1.37M
KCl	27mM
D-Glucose	55mM

1x Tyrode's

Dilute 10x Tyrode's 10 fold in distilled H_2O (pH must be 7.2-7.3, conductivity 13-15) and supplement with 5 mg/ml BSA

6.3.1b Procedure

Blood was withdrawn from healthy volunteers into ACD at a ration of 6:1, blood:ACD, using a 19G butterfly needle (Terumo), mixing intermittently during collection. After collection, each syringe was mixed thoroughly by inversion. Blood was transferred to 50 ml tubes containing 20 U/ml final Clexane and incubated at 37 ºC for 15 min. Blood was then centrifuged at 300 X g for 16 min, with brake level = 0. The top layer of platelet rich plasma (PRP) was transferred to new tubes, rested at 37ºC for 10 min then centrifuged at 1700 X g for 7 min, brake level = 7. The platelet poor plasma was then removed using a vacuum pump, and the platelet pellet resuspended in 1 x PWB + Clexane (20 U/ml final) to a volume equal to the original volume of PRP. The platelet count was checked, and maintained at ~3 x 10^8/ml. Platelets were rested for 10 min at 37 ºC, then centrifuged at 1500 x g for 7 min, brake level = 7. The platelet pellet was resuspended in 1 x Tyrode's buffer (pH 7.3) containing BSA (5mg/ml), calcium (1 mM) and Apyrase (0.02 U/ml). Platelets were rested for 30 min prior to use in further experiments.

6.3.2 Platelet Aggregation

Prior to use in further experimentation, platelet aggregation following a concentration curve of both ADP and collagen was measured to ensure platelet activation had not occurred during the washing procedure. Platelet aggregation was measured as previously described (Yap et al., 2000). In brief, 500 µg/ml fibrinogen was added to ~1.2 x 10^8 platelets/ reaction in a total volume of 400ul. Light emission readings for these resting platelets were measured for 20 s prior to the addition of agonist. ADP was added to final concentrations of 1, 2, 5 and 10 µM, while collagen final concentrations included 0.5, 1 and 10 µg/ml. Following stimulation, light transmission was observed for 10 min.

To investigate the effects of SK1 on platelet aggregation, SK-I and DMS were added to the washed platelets to final concentrations of 5µM each, and incubated for 30 min at 37 ºC

prior to addition of fibrinogen and agonists. The $S1P_2$ antagonist JTE-013 was added to a final concentration of 1 µM and treated as described above. The affect of each inhibitor was measured with both minimal and maximal agonist stimulation. For collagen, this was using 0.5 and 10 µg/ml, while ADP was added to final concentrations of 1 and 10 µM.

6.3.3 Fibrinogen Binding Assay

Washed platelets were diluted to a final concentration of 3 x 10^7 platelets/ ml in Tyrode's buffer. The platelets were incubated with 5 µM SK-I, 5 µM DMS, 1 µM JTE-013 or vehicle control for 30 min at 37 ºC. 20 µg/ml Oregon-Green Fibrinogen (OG-FNG) was added to this platelet suspension, and 50 µl platelets aliquoted into an eppendorf tube for each sample. The appropriate agonist was added per tube, and incubated for a further 10 min. Each reaction was stopped by dilution with 500 µl filtered PBS. OG-FNG binding was analysed by flow cytometry. Platelets were gated on Forward scatter *vs* side scatter to isolate healthy platelets, with this isolated population then gated for OGN fluorescence, with resting platelets used to control for background fluorescence. Agonists included 1 µM and 10 µM ADP, 1 µg/ml and 10 µg/ml CRP, and 1 µM and 10 µM TRAP.

6.4 Results

6.4.1 SK Inhibitors Attenuate Collagen-Induced Platelet Aggregation

The measure of platelet aggregation through the use of an aggregometer gives an indication of both the initial platelet shape change resulting from stimulation (inside-out signalling), observed by a drop in light transmission, in addition to subsequent aggregation (giving a measure of outside-in signalling), observed by a peak in light transmission. Prior to further experimentation, washed human platelets were stimulated with a range of ADP and collagen concentrations to ensure platelet activation had not occurred during the washing process (Figures 6.1A and 6.1B respectively). One can observe the transient nature of aggregate formation at low level stimulation, with aggregates forming and dissociating with time. High level stimulation however produced irreversible platelet aggregation.

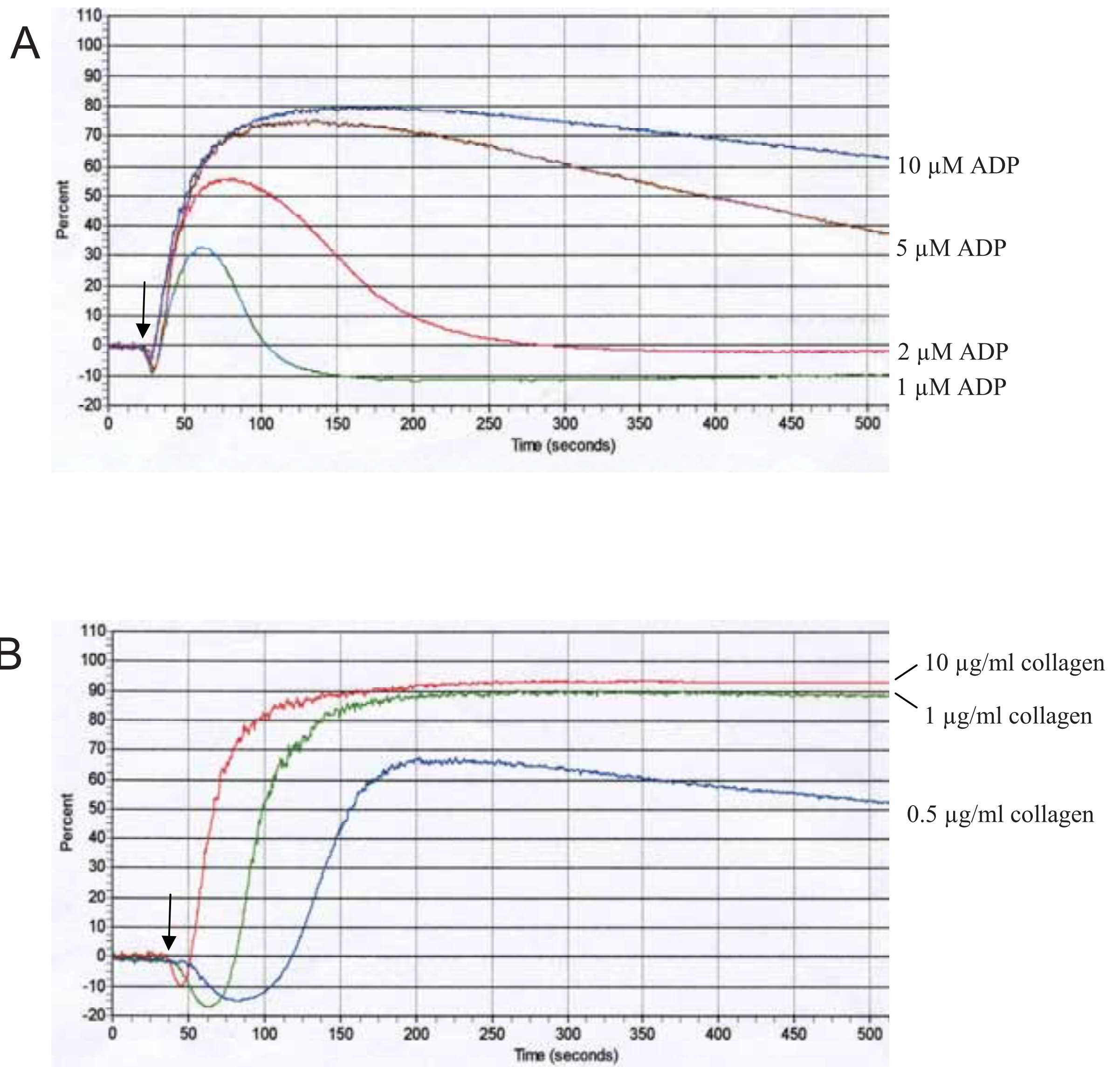


Figure 6.1. Platelet aggregation upon increasing concentrations of agonist stimulation
After 20 s of light transmission measurement, resting human platelets were stimulated with increasing concentrations of either (A) ADP or (B) collagen (arrow) and platelet aggregation observed for 10 min. Both the initial platelet shape change (characterised by an immediate dip in light transmission following agonist addition) in addition to aggregate formation (observed by a peak in light transmission) can be observed.
Results are representative of 3 independent experiments

Previous studies have demonstrated the ability of DMS to inhibit platelet activation (Yatomi et al., 1996). To recapitulate these results, and also examine the specific effect of SK1 in platelet activation, I employed the two SK inhibitors, DMS and SK-I and examined their effects on platelet aggregation induced by both maximal and sub-maximal collagen stimulation. Washed human platelets were incubated with fibrinogen and either 5 μM DMS, 5 μM SK-I or DMSO vehicle control for 30 min, prior to aggregate measurement upon collagen stimulation. From these results (Figure 6.2), it was apparent that both DMS and SK-I inhibited the low-dose collagen-mediated platelet aggregation, although the high dose aggregation remained unaffected. This was observed by the lack of a high peak in light transmission in the inhibitor treated platelets upon the low dose collagen stimulation. However, these inhibitor treated platelets still underwent an initial shape change, observed by the initial drop in light transmission, which was comparable to the vehicle alone treated cells. Hence, it appeared that SK1 inhibition likely affects outside-in signalling, but not the prior inside-out signalling events mediating platelet aggregation.

6.4.2 SK Inhibitors do not affect ADP-Induced Platelet Aggregation

In order to determine whether the affects of the SK1 inhibitors were specific to collagen-mediated platelet activation, following washing and incubation with SK-I and DMS (as described above), platelets were activated with both low and high dose ADP, and aggregate formation observed. These results (Figure 6.3) showed that neither DMS nor SK-I affected low nor high dose platelet aggregation upon stimulation with ADP. Hence, it appeared that the affect of SK1 inhibition on platelet aggregation may indeed be a specific to stimulation by collagen.

6.4.3 Inhibition of the S1P Receptor $S1P_2$ Inhibits Collagen-Specific Platelet Aggregation

Activated platelets release S1P into the plasma, which can then activate neighbouring platelets in a feed-forward manner, ultimately resulting in platelet aggregation (Yatomi et al., 1995b). While the S1P receptors $S1P_1$, $S1P_2$ and $S1P_3$ are the major subtypes expressed on endothelial cells, cardiomyocytes and smooth muscle cells, $S1P_2$ has been

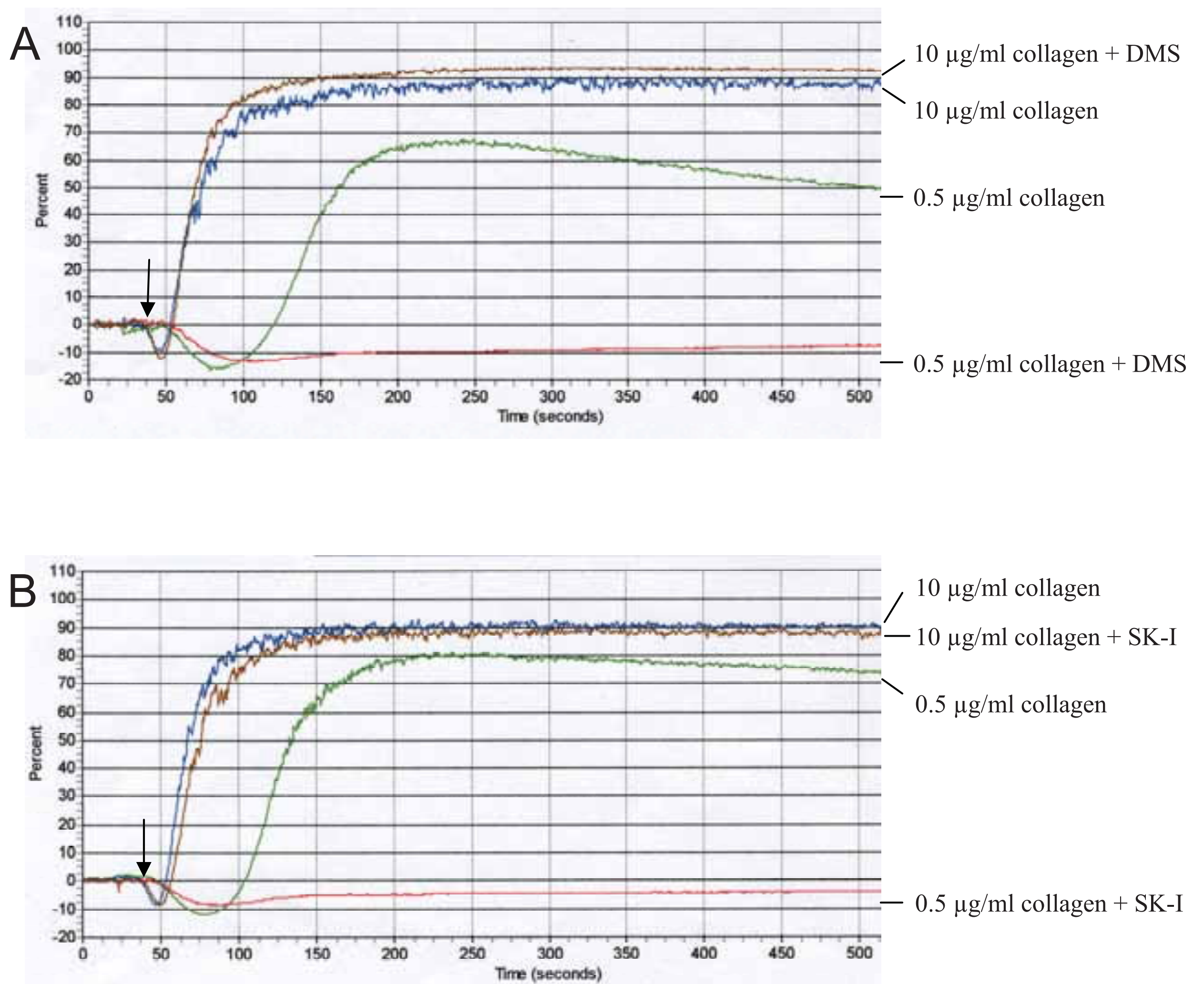


Figure 6.2. SK inhibition prevents collagen-mediated platelet aggregation

(A) Platelets were incubated with either 5 µM DMS or DMSO for 30 min, prior to 0.5 µg/ml or 10 µg/ml collagen stimulation and aggregation observed for 10 min. The arrow shows agonist addition.

(B) Platelets were incubated with either 5 µM SK-I or DMSO for 30 min, prior to 0.5 µg/ml or 10 µg/ml collagen stimulation and aggregation observed for 10 min. The arrow shows agonist addition.

Results are representative of 3 independent experiments

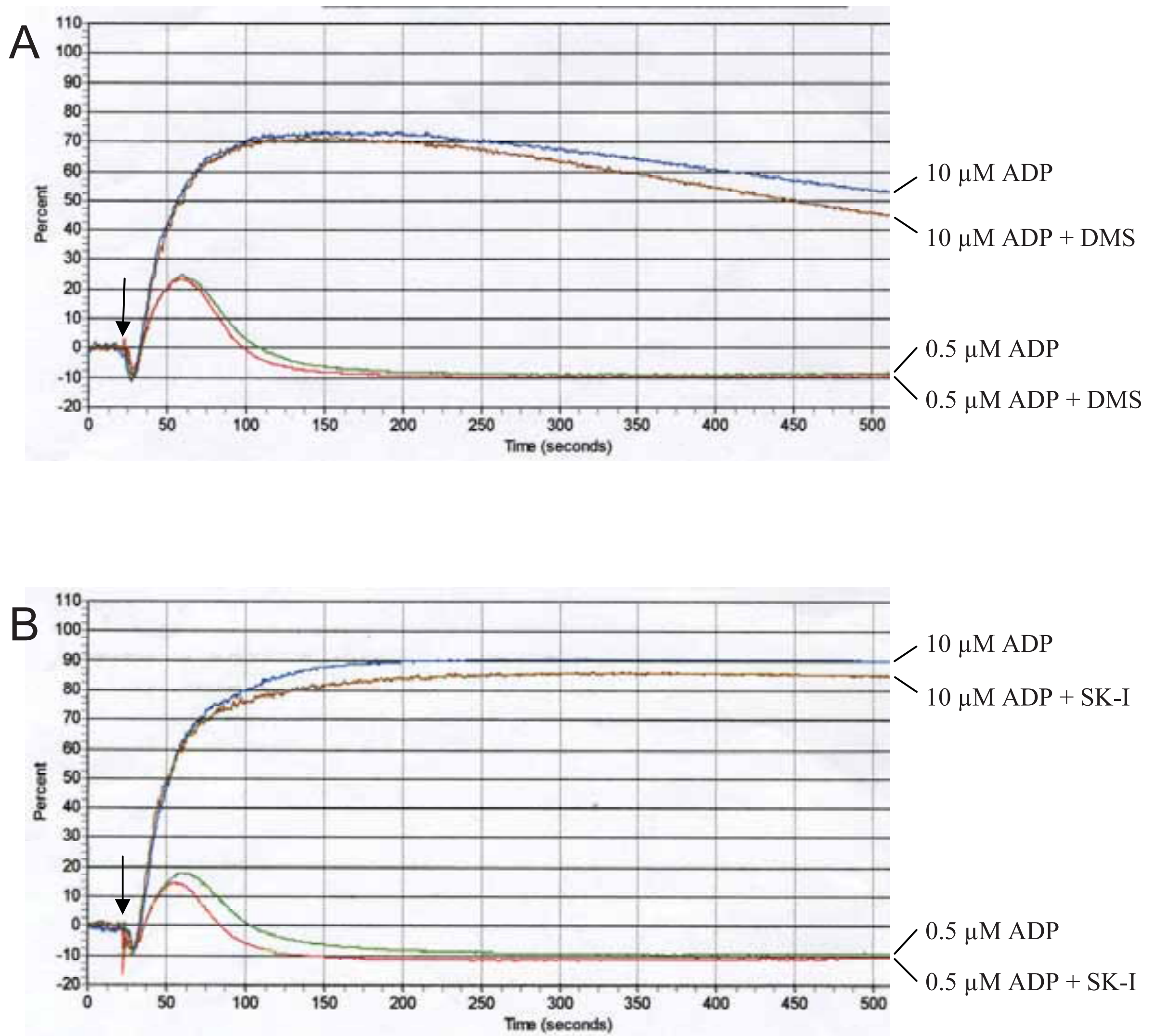


Figure 6.3. SK inhibition has no effect on ADP-mediated platelet aggregation

(A) Platelets were incubated with either 5 μM DMS or DMSO for 30 min, prior to 0.5 μM or 10 μM ADP stimulation and aggregation observed for 10 min. The arrow shows agonist addition.

(B) Platelets were incubated with either 5 μM SK-I or DMSO for 30 min, prior to 0.5 μM or 10 μM ADP stimulation and aggregation observed for 10 min. The arrow shows agonist addition.

Results are representative of 3 independent experiments

shown to be the main S1P receptor expressed on human platelets (Randriamboavonjy et al., 2009). Antagonism of $S1P_2$ has also been shown to attenuate both platelet aggregation and increases in intracellular Ca^{2+} upon S1P exposure (Randriamboavonjy et al., 2009). To determine whether the $S1P_2$ receptor on the platelet surface may also be involved platelet activation induced by other agonists, I pre-treated washed human platelets with the $S1P_2$ antagonist JTE-013 prior to stimulation with collagen or ADP and observed aggregation. Similar to the results achieved using the SK1 inhibitors, inhibition of $S1P_2$ specifically blocked the low dose collagen-mediated platelet aggregation (Figure 6.4A). Again, JTE-013 specifically affected the outside-in signalling event, with the initial shape change upon stimulation unaffected. Again, however, neither the high nor low dose ADP-induced platelet activation was affected by JTE-013 (Figure 6.4B).

6.4.4 Evaluation of Fibrinogen Binding upon SK Inhibition

As a further measurement of platelet activation I utilised a fibrinogen binding assay to determine the affects of the SK inhibitors on this outside-in signalling event. Fibrinogen binding was measured after stimulation with maximum and sub-maximal stimulation with the following agonists: ADP; CRP (collagen-related peptide); and TRAP (thrombin receptor-activating protein). Washed human platelets were incubated with SK-I or DMS prior to addition to Oregon-Green Fibrinogen (OG-FNG) and the appropriate agonist. The amount of OG-FNG bound to these platelets was quantitated by flow cytometry, using resting platelets to control for non-specific fluorescence.

With this assay, it was quite apparent that there was very little difference between the results from each of the low versus high agonist stimulations (Figure 6.5A and B). With each agonist examined, DMS was able to completely inhibit fibrinogen binding (Figure 6.5). This was quite unexpected, and so I microscopically examined DMS treated platelets. These platelets displayed a very rounded, abnormal morphology, very unlike control platelets. Hence, it seems likely that DMS had a non-specific affect on these platelets, making it difficult to ascertain the direct effect of this inhibitor on fibrinogen binding.

The SK-I treated platelets behaved quite differently to those subjected to DMS. In the ADP stimulated platelets, SK-I reduced fibrinogen binding to approximately 50% of the control

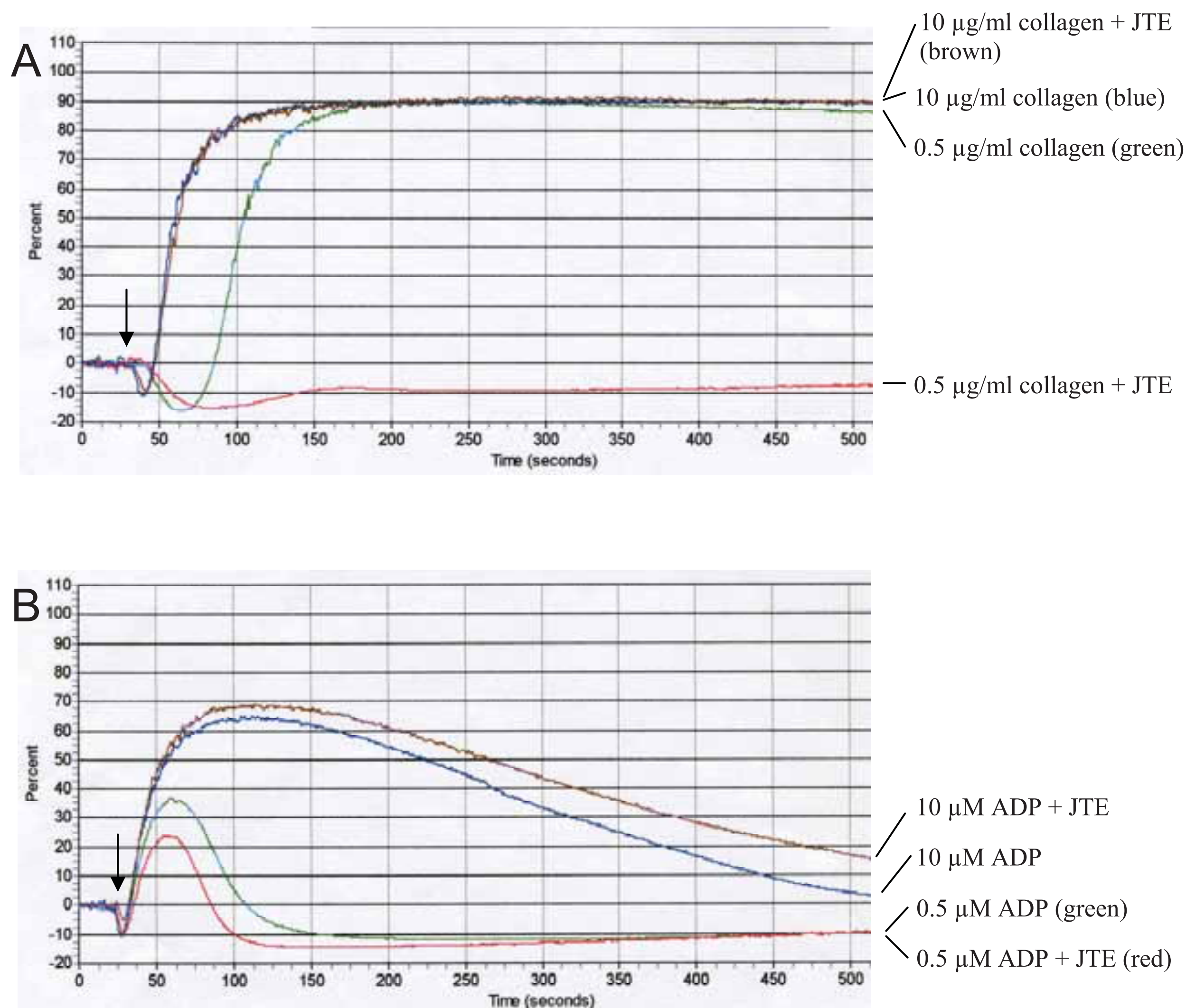


Figure 6.4. $S1P_2$ antagonism prevents collagen-mediated platelet aggregation

(A) Platelets were incubated with either 1 μM JTE-013 (JTE) or vehicle for 30 min, prior to 0.5 μg/ml or 10 μg/ml collage stimulation and aggregation observed for 10 min. The arrow shows agonist addition.

(B) Platelets were incubated with either 1 μM JTE-013 or vehicle for 30 min, prior to 0.5 μM or 10 μM ADP stimulation and aggregation observed for 10 min. The arrow shows agonist addition.

Results are representative of 3 independent experiments

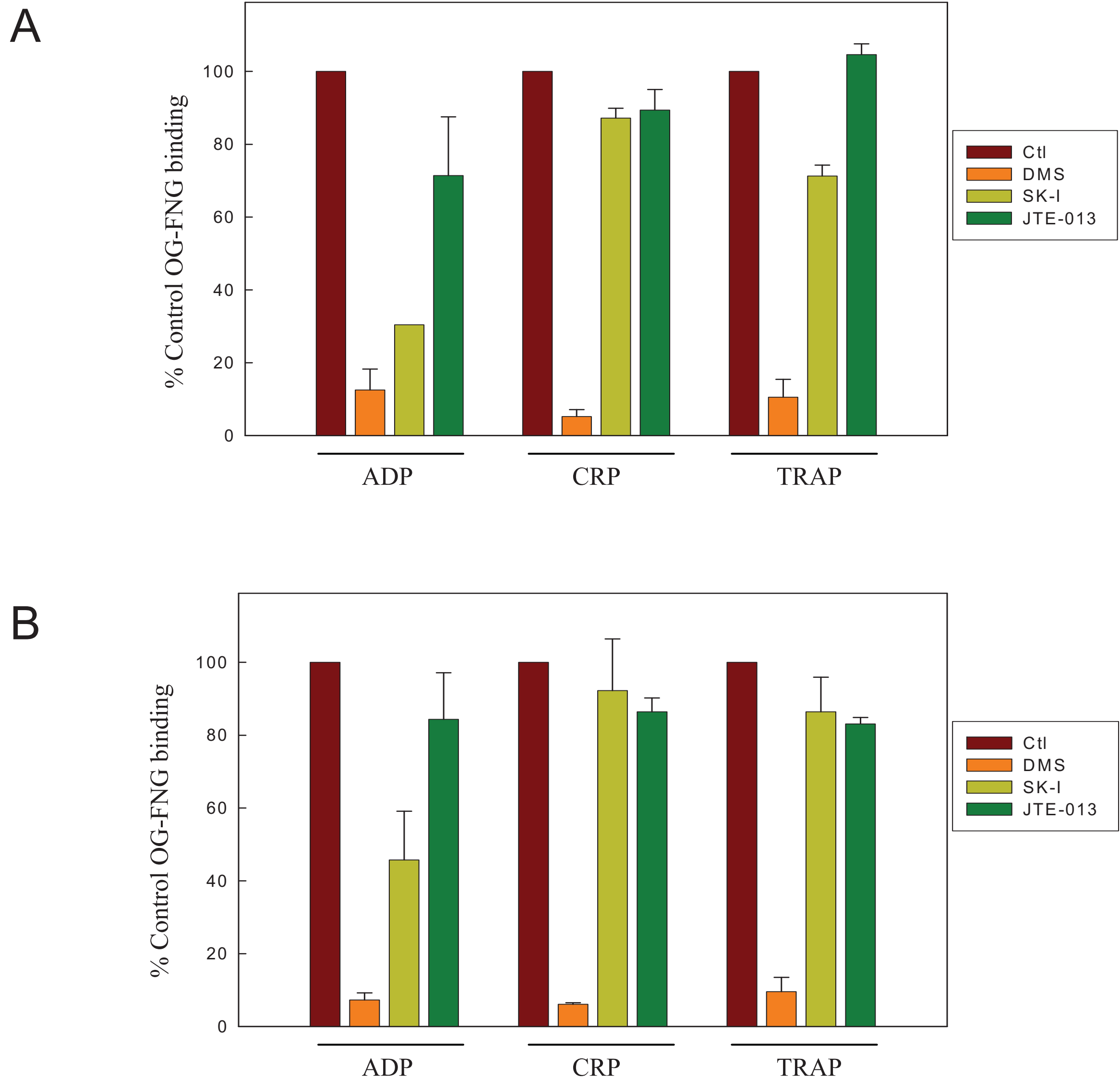


Figure 6.5. The effects of DMS, SK-I and JTE-013 on Oregon-green Fibronogen binding following high and low agonist stimulations.

Platelets were incubated with 5 µM SK-I, 5 µM DMS, 1 µM JTE-013 or vehicle control (ctl) for 30 min prior to Oregon-Green Fibrinogen (OG-FNG) addition and agonist stimulation. OG-FNG binding was analysed by flow cytometry, with results here describing the percentage of OG-FNG bound compared to the control.

(A) OG-FNG binding following low agonist stimulation as follows: 1 µM ADP, 1 µg/ml CRP, and 1 µM TRAP.

(B) OG-FNG binding following high agonist stimulation as follows: 10 µM ADP, 10 µg/ml CRP, and 10 µM TRAP.

Data shows mean of 2 independent experiments ± range.

platelets. However, only very modest effects, if any at all were observed with those stimulated with CRP and TRAP (Figure 6.5). This was somewhat surprising, considering the previous data showing opposite affects to these of SK-I on ADP and collagen mediated platelet aggregation.

6.4.5. Evaluation of Fibrinogen Binding upon $S1P_2$ Antagonism

In order to determine the role of the $S1P_2$ receptor in the process of fibrinogen binding to activated platelets, the inhibitor JTE-013 was used in OG-FNG binding assays as described above. Results showed that only very modest differences were observed between the JTE-013 and control platelets upon stimulation with each of ADP, CRP and TRAP (Figure 6.5). Hence, it seemed unlikely that $S1P_2$ has a role in fibrinogen binding to platelets activated through these pathways.

6.5 Discussion

Platelet activation and subsequent aggregation is critical in processes such as wound healing and the maintenance of vascular integrity. Although previous studies have established a role for S1P in platelet aggregation (Yatomi et al., 1995a; Yatomi et al., 1997), limited information is available on the direct function of SK1 in this process.

In this study, I have examined the affects of the SK inhibitors DMS and SK-I on platelet aggregation and fibrinogen binding. While the ADP- induced platelet aggregation remained unaffected, both of these inhibitors prevented the low dose platelet aggregation upon collagen stimulation. This suggested that SK1 may be required for specific signalling events upon collagen activation, with these not critical in ADP-induced platelet activation.

The exposure of collagen to platelets confers a dual effect; first in acting as a substrate for platelet adhesion, as well as inducing platelet activation. The prevailing evidence proposes that two receptors are involved in the platelet response to collagen; integrin $\alpha II\beta 1$ acts to adhere platelets to collagen, while the lower affinity receptor glycoprotein VI (GPVI), is mainly responsible for platelet activation (Roberts et al., 2004a). While most platelet

agonists including ADP and thrombin stimulate their own G protein-coupled receptors, activating PLCβ through G_q, GPVI reportedly tyrosine-phosphorylates and activates PLCγ2 (Ohmori et al., 2005). Hence, the collagen-specific effect of SK inhibition may be a result of these varied signalling pathways.

Despite the lack of aggregate formation, the inhibitor treated platelets were able to undergo an initial shape change upon collagen stimulation. This suggested that the inside-out signalling pathway is still intact in SK inhibited platelets, with the defect leading to a lack of aggregation occurring subsequent to this. This notion is supported by previous studies showing the tyrosine phosphorylation of Syc and PLCγ2 by GPVI remain unaffected by DMS (Ohmori et al., 2005).

Surprisingly, these results were not completely consistent with the ability of the SK inhibitor treated platelets to bind fibrinogen. Firstly, the use of DMS in this assay appeared to affect the health of these platelets. In comparison to vehicle treated platelets, those subjected to DMS displayed an abnormal morphology, and were not capable of binding fibrinogen following stimulation with any agonist tested. Hence, it was concluded that DMS likely has a non-specific affect on these platelets. This was not overly surprising considering DMS is not a specific SK inhibitor, with this lipid also affecting a number of other signalling enzymes (Woodcock, 2006).

In contrast to DMS, SK-I did not appear to have a deleterious effect on the morphology of platelets. SK-I treatment of TRAP-activated platelets did not have any significant affect on the ability of these platelets to bind fibrinogen. This was not surprising considering prior studies showing that SK inhibition does not affect thrombin induced intracellular signalling such as Ca^{2+} mobilisation in platelets (Ohmori et al., 2005; Yang et al., 1999). One quite unexpected result, however, was the lack of any significant inhibitory affect of SK-I on the ability of CRP (a collagen receptor agonist) stimulated platelets to bind fibrinogen. In platelet activation, the binding of fibrinogen occurs after the initial inside-out signalling event, but prior to aggregate formation. Hence, together with the aggregation results, it is possible that both the initial platelet shape change and the binding of fibrinogen to the collagen-activated platelets remain intact, with SK inhibition affecting signalling subsequent to these events to affect the final platelet aggregation.

Although a number of studies have observed no overall change in SK1 activity following platelet stimulation (Yatomi et al., 1997; Ohmori et al., 2005), Ohmori and colleagues (2005) demonstrated an increase in SK1 activity in the membrane fraction of platelets when specifically activated through the collagen receptor. These authors hypothesized that re-localisation of SK1 to the platelet membrane, specific to collagen stimulation may function to generate a localised pool of S1P close to the platelet membrane and produce the differential responses observed with a variety of other agonists upon DMS treatment. Hence, it may be possible that the inhibition of SK may attenuate this localised production of S1P, with this event critical in collagen-mediated platelet aggregation. As S1P released from activated platelets is predicted to result in a feed-forward activation of neighbouring platelets, the inhibition of this localized pool of S1P may be the source of the defective aggregation. As CIB1, the protein responsible for the plasma membrane translocation of activated SK1 in mammalian cells (Chapter 3) is expressed in human platelets, it would be interesting to determine whether this role of CIB1 in SK1 translocation is conserved in platelets. This may function to generate the localized pool of S1P at the plama membrane, critical for platelet aggregation, but not required for initial shape-change nor fibrinogen binding.

Despite SK-I affecting neither TRAP nor CRP-induced fibrinogen binding, this inhibitor quite surprisingly reduced the ability of fibrinogen to bind ADP-activated platelets. This is in direct contrast to the aggregation results generated in this study, where SK-I had no affect on both low and high dose ADP-dependent platelet aggregation. It is also in contrast to prior studies where SK inhibition had no affect on ADP-induced Ca^{2+} mobilisation (Ohmori et al., 2005; Yang et al., 1999). Hence, the ability of SK-I to inhibit fibrinogen binding, yet not the initial shape change nor aggregation resulting from ADP stimulation is quite confusing, with further experimentation required to clarify these results.

The release of S1P from activated platelets is thought to further activate neighbouring platelets in a feed-forward manner, resulting in localised aggregation and clot formation. Randriamboavonjy and colleagues (2009) identified the S1P receptor, $S1P_2$ as being the main S1P receptor expressed on human platelets. Here, I have identified a role for $S1P_2$ in the collagen-mediated platelet aggregation with this receptor antagonist JTE-013 specifically inhibiting the low-dose collagen induced platelet aggregation. This inhibitor did not affect the initial platelet shape change or the ability of the activated platelets to bind

fibrinogen with all agonists tested, suggesting that the $S1P_2$ receptor functions downstream of these events in collagen-induced platelet activation. This is consistent with a role for $S1P_2$ in the feed-forward activation of neighbouring platelets upon exposure to secreted S1P. Notably, these results are consistent with a previous study in which $S1P_2$ antagonism inhibited the S1P-induced platelet aggregation, and modestly attenuated the S1P-induced increase in intracellular Ca^{2+} (Randriamboavonjy et al., 2009).

Overall, this study has identified a specific role for both SK1 and $S1P_2$ in collagen-mediated platelet aggregation. As inhibition of each of these affected neither platelet shape change nor fibrinogen binding, it is likely that both have a critical role in platelet aggregation subsequent to these events.

Chapter 7: General Discussion

7.1 Aims of this Study

SK1 plays important roles in many aspects of cellular regulation. While the 'housekeeping' role for this enzyme in the sphingomyelin cycle has been proposed to balance cellular levels of its upstream pro-apoptotic mediators, ceramide and sphingosine, with the pro-survival and pro-proliferative signalling of its product S1P (Spiegel and Milstien, 2003b), SK1 can also be transiently activated by a number of agonists at the cell surface generating a greater supply of S1P. This S1P has been shown to have many diverse signalling functions, leading to the identification of de-regulated SK1 activity in many patho-physiological conditions, including cancer.

The transient activation of SK1 induced by a number of agonists at the cell surface is regulated through phosphorylation of the enzyme at Ser225 by ERK1/2 (Pitson et al., 2003; Pitson et al., 2005). Following phosphorylation, SK1 is translocated from the cytosol to the plasma membrane, with both of these events critical in SK1 induced tumourigenesis (Pitson et al., 2005). The translocation of SK1 to the plasma membrane is hypothesised to bring SK1 into close proximity to sphingosine stores generated at the plasma membrane and engender a localised production of S1P. This S1P can be released from the cell, or could possibly activate downstream intracellular targets. Although critical in understanding SK1 induced oncogenesis, the mechanisms regulating the rapid agonist-induced translocation of SK1 to the plasma membrane are poorly understood. The general aims of this study were to identify the protein/s responsible for translocating SK1 to the plasma membrane upon agonist stimulation, with the future hope of manipulating this event for cancer therapeutics.

7.2 CIB1 Mediates SK1 Translocation and Oncogenic Signalling

Prior to this study, the molecular mechanism mediating the agonist-induced translocation of SK1 to the plasma membrane remained undefined. One recent study hypothesised the actin crosslinking protein Filamin A (FLNa) to be involved in the Heregulin (Hrg)-induced localisation of SK1 to the lamellipodia in A7 melanoma cells (Maceyka et al., 2008).

However, as ERK1/2 mediated SK1 phosphorylation did not appear to play a role in the lamellipodia-localisation of SK1 in this system, it is possible that multiple translocation mechanisms may exist which vary dependent upon cell type and the SK1 activating agonist. Others studies have indirectly implicated Calmodulin (CaM) in the translocation of this enzyme. This evidence stems from experiments where both the use of a CaM inhibitor and mutation of the CaM-binding site of SK1 were able to block SK1 translocation (Young et al., 2003; Sutherland et al., 2006). However, evidence for a direct role for CaM in SK1 translocation had not been described. In addition, the inability to co-immunoprecipitate SK1 with CaM from cells, even following overexpression of both proteins (Pitson laboratory, unpublished data), questions the ability of these two proteins to interact in cells. Furthermore, the predominant movement of CaM to the nucleus, not the plasma membrane, in response to cellular Ca^{2+} fluxes (Chin and Means, 2000; Thorogate and Torok, 2007) raised doubts over the role of CaM in SK1 localisation.

In this study, I have identified the CaM-related protein, CIB1 as mediating the agonist-induced translocation of SK1 to the plasma membrane. By targeting CIB1 through siRNA mediated knock-down of CIB1 expression or through the use of a dominant-negative CIB1 mutant, I was able to ablate the agonist-induced translocation of SK1 to the plasma membrane. This silencing/inhibition of CIB1 had no effect on ERK1/2 phosphorylation, supporting a direct requirement of CIB1 in mediating the translocation of SK1.

The ability of CIB1 to interact with SK1 in the previously identified CaM binding site was not surprising, considering the 55 % amino acid similarity, as well as structural similarity these two proteins share. CIB1 is a 22 kDa alpha-helical protein composed of four EF-hands (Yamniuk et al., 2004; Gentry et al., 2005; Yamniuk and Vogel, 2005; Blamey et al., 2005; Hwang and Vogel, 2000). Structural characterisation of CIB1 has shown this protein to bind Ca^{2+} into its two C-terminal EF hands, resulting in a significant conformational change (Yamniuk et al., 2004). This functions to both stabilise the protein, in addition to revealing a hydrophobic binding channel spanning the entire C- and part of the N-terminus of the protein (Yamniuk et al., 2004; Yamniuk and Vogel, 2006). Interestingly, not only is this hydrophobic channel conserved in CaM, but both CIB1 and CaM appear to target analogous alpha-helical regions on target proteins (Barry et al., 2002).

The ability of the 'CaM inhibitor' W7 to block the CIB1-SK1 interaction in this study was somewhat surprising, considering its historical status as a CaM-specific inhibitor. However, upon examination of the mechanism by which W7 binds and inhibits CaM, it is not overly surprising that CIB1 too, could be bound by this compound. W7 binds into the hydrophobic channel of Ca^{2+}-bound CaM through interactions between its naphthalene ring and the hydrophobic residues in CaM's protein-binding pocket (Osawa et al., 1998). 22 residues in CaM are required for interacting with W7, and through amino acid comparison, 18 of these are conserved in CIB1 (Figure 7.1). Hence, this high level of conservation likely enables the inhibitory effect of W7 on CIB1. Notably, W7 can only bind Ca^{2+}-bound CaM (Osawa et al., 1998), and this is likely the case also with CIB1, with this protein structurally similar to CaM following conformational change upon binding Ca^{2+} (Yamniuk et al., 2004). This cross-reactivity of W7 has become more evident recently, with the identification of its ability to also bind cardiac troponin and inhibit striated muscle contraction (Hoffman et al., 2005; Hoffman and Sykes, 2009; Li et al., 2006). The ability of W7 to inhibit proteins other than CaM is somewhat concerning given the number of cellular functions attributed to this protein through the use of this inhibitor (Tanaka, 1988). Due to this evidence, it seems likely that the lack of SK1 translocation observed upon treatment of cells with W7 (Young et al., 2003) is due to the targeting of the CIB1-SK1 interaction.

Upon activation of SK1, increases in cytosolic Ca^{2+} levels have been widely observed (Spiegel and Milstien, 2003b). Interestingly, both CIB1 (this study) and CaM (Pitson et al., 2000a; Sutherland et al., 2006) interact with SK1 in a Ca^{2+}-dependent manner. In light of this, it is notable that the affinity of CIB1 for Ca^{2+} is just above basal cellular concentrations (K_d = 0.54 μM), and approximately ten-fold higher than that of CaM (Yamniuk et al., 2004). Although the physiological interaction of CaM with SK1 has not been established, if this interaction indeed does occur, CIB1 and CaM likely compete for binding to SK1. Hence, in addition to differential binding affinities, the Ca^{2+} concentration in the cell seems a plausible regulator of these interactions. Hence, it is of note that CIB1 is able to interact with SK1 in an environment with as low as 0.5 μM Ca^{2+}, a concentration at which CaM would remain inactive. This difference in Ca^{2+} affinity likely provides selectivity for activation of CIB1 alone upon certain agonist stimulations. Of particular note phorbol ester stimulation, which induces robust translocation of SK1 to the plasma membrane, is only a weak inducer of Ca^{2+} fluxes (Murphy et al., 1994). Hence, stimulation by this agonist likely activates CIB1 in the absence of CaM activation.

```
CIB1          MGGSGSRLSKELLAEYQDLTFLTKQEILLAHRRFCELLPQEQRTVESSLRAQVPFEQILS 60
Calmodulin    ---MADQLTEEQIAEFKEAFSLFDKD------GDGTITTKELGTVMRSLGQNP------- 44

CIB1          LPELKANPFKERICRVFSTSPAKDSLSFEDFLDLLSVFSDTATPDIKSHYAFRIFDFDDD 120
Calmodulin    ----TEAELQDMINEVDAD--GNGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGN 98

CIB1          GTLNREDLSRLVNCLTGEGEDTRLSASEMKQLIDNILEESDIDRDGTINLSEFQHVISRS 180
Calmodulin    GYISAAELRHVMTNLG-----EKLTDEEVDEMIR----EADIDGDGQVNYEEFVQMMTAK 149

CIB1          PDFASSFKIVL 191
Calmodulin    -----------
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Figure 7.1. Amino acid residues in CaM required for W7 binding, conserved in CIB1
Sequence alignment of CIB1 with CaM (Calmodulin) showing the residues in calmodulin required for interaction with W7 (black box, red writing). Identical residues in CIB1 are similarly displayed, while conserved residues are also highlighted (black box, white writing)

In addition to binding Ca^{2+}, CIB1 has also been identified to bind Mg^{2+} in EF3 with a weak affinity (K_d = 120 μM; Yamniuk et al., 2004; Yamniuk and Vogel, 2005). This binding of Mg^{2+} has been shown to induce similar structural changes in CIB1 to that of Ca^{2+} and also enable the binding of CIB1 to αIIb integrin cytoplasmic domain peptides *in vitro* (Yamniuk and Vogel, 2005). Mg^{2+}, however, was not able to substitute for Ca^{2+} in enabling the interaction between CIB1 and SK1. As Mg^{2+} is present in millimolar concentrations inside the cell, it is likely that EF3 of CIB1 is basally occupied with this metal ion (Yamniuk et al., 2004; Yamniuk and Vogel, 2005). Increased cytosolic Ca^{2+} levels are predicted to cause the low affinity binding of Mg^{2+} to be displaced by its higher affinity association for Ca^{2+} (Yamniuk et al., 2004). Hence, we propose a model by which activation of SK1 and the Ca^{2+} flux associated with this event cause the Mg^{2+} bound to EF3 to be replaced by a higher affinity association with Ca^{2+}, activating CIB1. This would then enable the interaction between CIB1 and SK1, and allow CIB1 to shuttle SK1 to the plasma membrane.

Quite surprisingly, CIB1 was able to interact with both phosphorylated and non-phosphorylated SK1. This is despite non-phosphorylated SK1 not appearing to localise to the plasma membrane upon agonist stimulation (Pitson et al., 2005). However, phosphatidylserine, a plasma membrane localised phospholipid has been shown to preferentially bind phosphorylated SK1 (Stahelin et al., 2005), likely mediating retention of phosphorylated SK1 at the plasma membrane. Thus, although CIB1 may be able to shuttle both phosphorylated and non-phosphorylated SK1 to the plasma membrane, it is likely that only phosphorylated SK1 is retained here, via association with this phospholipid. It is also possible that other still unknown mechanisms may operate to regulate the phosphorylation-specific translocation of SK1 *in vivo*. One possibility is that non-phosphorylated SK1 may specifically interact with other proteins, which act to 'anchor' it in the cytoplasm and prevent it from translocating to the plasma membrane. The phosphorylation of SK1 could break this interaction, and allow CIB1 to function to carry phosphorylated SK1 to the plasma membrane.

In addition to Ser225, a second potential phosphorylation site has been identified in SK1 at Thr193 by a mass spectroscopy approach using HeLa cell lysates (Dephoure et al., 2008). This phosphorylation was detected on endogenous SK1 after cell cycle arrest at mitosis (Dephoure et al., 2008). Hence, the physiological relevance of this phosphorylation is yet to

be determined. However, as this phosphorylation site is close to the critical residues involved in the CIB1-SK1 interaction (Phe197 and Leu198), future studies should examine whether phosphorylation of this site firstly; regulates the CIB1-SK1 interaction, and secondly; influences the subsequent agonist-induced translocation of SK1 to the plasma membrane.

In this study, I demonstrated the ability of CIB1 to function as a Ca^{2+}-myristoyl switch, translocating to the plasma membrane upon ionomycin, thapsigargin or PMA stimulation in a myristoyl and Ca^{2+} dependent manner. Hence, this provides a functional mechanism by which CIB1 can actively transport SK1 to the plasma membrane upon activation by Ca^{2+}. As CIB1 is quite widely expressed (Shock et al., 1999) and has been identified as interacting with a number of targets inside cells (reviewed in Yamniuk and Vogel, 2006), it is thought that CIB1 may have a wider regulatory role in cell signalling pathways. The finding of the Ca^{2+}-myristoyl switch behaviour of CIB1 is the first identification of a mechanism by which it may exert its regulatory affects on its various targets within the cell. In line with this mode of action, Myo1c, a membrane associated member of the myosin superfamily has been previously identified as a CIB1 interactor (Tang et al., 2007). This protein also binds CaM but competes for binding to CIB1 in the presence of Ca^{2+}. Myo1c has roles in membrane dynamics, cytoskeletal structure, mechanical signal-transduction, and membrane trafficking, with a proposed role for CIB1 in determining its sub-cellular localisation (Tang et al., 2007). Hence, the Ca^{2+}-myristoyl switch activity of CIB1 may function to facilitate this membrane localisation. At least two other CIB1 target proteins, Presinillin 2 and the αIIb integrin are membrane localised. Hence, it would be interesting to determine whether CIB1 may regulate the function of these interacting partners through the recruitment and delivery of additional regulatory proteins to these membrane localised targets.

The biological significance of the CIB1 mediated SK1 translocation was demonstrated in this study by the ability of CIB1 siRNA to significantly reduce the agonist-induced production of S1P. In addition, both CIB1 siRNA and the expression of a dominant-negative, non-myristoylated CIB1 were able to block the TNF-α associated survival signalling pathway, through attenuation of NFκB activation. SK1 activity has previously been shown to be one of the critical factors facilitating this anti-apoptotic signalling (Xia et al., 1999b). Hence, these results suggest a requirement for SK1 translocation to the plasma membrane (mediated by CIB1) for these survival signalling events. To provide further

evidence that this role of CIB1 in the TNF-α associated survival pathway is mediated directly through its function in SK1 translocation, future studies should examine whether the expression of a variant of SK1 that is constitutively localised to the plasma membrane (Pitson et al., 2005) can rescue the TNF-α mediated survival pathway in CIB1 knock-down cells.

As previously described, both the phosphorylation and subsequent translocation of SK1 to the plasma membrane are critical for SK1-mediated neoplastic transformation (Pitson et al., 2005). The ability of CIB1 expression in NIH3T3 fibroblasts to result in foci formation was exciting, as it is the first indication that CIB1 itself may have oncogenic potential. As inhibition of SK prevented the formation of these foci, one can assume the loss of contact inhibition in these cells is mediated directly through the SK signalling pathway. However, as focus formation assays give only an *in vitro* indication of oncogenic potential, further studies will need to be completed to determine the true oncogenic nature of CIB1. These should include the determination of whether CIB1 expression can result in colony formation in soft agar, in addition to subcutaneous injection of CIB1 expressing fibroblasts into nude mice to determine if these cells can result in tumour formation *in vivo*. These studies will more conclusively determine the ability of CIB1 to induce neoplastic transformation. Promisingly, examination of gene expression data from microarray analysis has shown an upregulation of CIB1 expression in multiple cancer types, when compared to matched normal tissues (Figure 7.2). This is most evident in ovarian cancer, where an elevation of CIB1 expression is evident in multiple tumour types (Hendrix et al., 2006), but also in invasive ductal breast carcinoma (Karnoub et al., 2007; Sorlie et al., 2003; Sorlie et al., 2001; Perou et al., 2000), lung adenocarcinoma (Garber et al., 2001; Beer et al., 2002) and superficial bladder cancer (Sanchez-Carbayo et al., 2006; Blaveri et al., 2005). These studies support our notion that deregulated CIB1 expression may lead to an oncogenic outcome.

The non-myristoylated CIB1 generated in this study was not only shown to no longer act in a Ca^{2+}-myristoyl switch manner, but its expression in cells was demonstrated to act in a negative fashion to block the translocation of SK1 to the plasma membrane. Hence, this non-myristoylated CIB1 appears to act in a dominant-negative fashion. However, it must be noted that while we refer to this CIB1 variant as a ‘dominant-negative’, this may not be completely accurate, as a true dominant-negative would have to bind SK1 with a higher affinity than endogenous/wildtype CIB1. This has not yet been tested. In addition to its

NOTE:
These figures are included on page 95a of the print copy of the thesis held in the University of Adelaide Library.

(Hendrix et al., 2006) (Karnaub et al., 2007)

Figure 7.2. CIB1 gene expression is elevated in some naturally occurring tumours
CIB1 gene expression compiled from microarray analysis from indicated cancer tissues, compared to matched normal tissue samples. Data shows the median, range between the 25th and 75th percentile (box) +/- SEM. The number of samples analysed for each cancer type is indicated in brackets. Oncomine™ (Compendia Bioscience, Ann Arbor, MI) was used for analysis and visualization.

inhibitory effect on SK1 translocation, expression of this non-myristoylated CIB1 also blocked the TNF-α mediated cell survival pathway. The ability of this CIB1 variant to block each of these downstream signalling events associated with SK1 activation and translocation suggests CIB1 to represent a promising target to manipulate SK1 signalling in the cell. Most remarkably, the non-myristoylated CIB1 was also able to attenuate neoplastic transformation induced by oncogenic Ras. SK1 activity has previously been demonstrated as important in Ras-mediated oncogenesis, with a dominant-negative SK1 and the use of the SK inhibitor DMS able to attenuate Ras transformation (Xia et al., 2000). The ability of the non-myristoylated CIB1 to block Ras transformation suggests the CIB1-mediated translocation of SK1 to the plasma membrane to be an integral part of the Ras oncogenic pathway. In addition to the potential transforming ability of CIB1 itself, these results raise the possibility of targeting the CIB1-SK1 interaction for cancer therapeutics. This will be discussed in more detail in section 7.5.2.

One potential limitation of this study was necessary use of genetic manipulations to study the function of CIB1 in the SK1 signalling pathway. CIB1 siRNA was employed to knockdown this protein in cells, while other experiments utilised overexpression of a mutated version of this protein to study the affect of CIB1 on SK1 function. As previously described, CIB1 has multiple interacting partners, and probably has roles in numerous signalling pathways. Hence, the genetic manipulations described above were likely to not only affect the function of CIB1 in respect to SK1 signalling, but also the other cellular functions of this protein. Hence, it is difficult to conclude whether some of the results measured in this study, such as the increase in TNF-α-induced apoptosis in CIB1 deficient cells, are caused directly through an altered SK1 signalling pathway, or are actually a result of another defective CIB1 function unrelated to SK1. Potential ways to overcome some of these limitations may involve 'rescue' experiments, where application of S1P or expression of a constitutively plasma membrane localised version of SK1 may help to determine the role of SK1 in the biologial effects of genetic manipulation of CIB1. Alternatively, the identification and mutation of the SK1 binding site on CIB1 may also assist in this area. The mutation of this binding site may only disrupt the CIB1-SK1 interaction and not affect other CIB1 functions. The development of a genetic mouse model containing this mutated CIB1 version could help to isolate specific CIB1-SK1 signalling functions, while minimising non-specific affects of CIB1 on other signalling pathways.

7.3 Does CIB1 Mediate other Signalling Events Associated with S1P Production?

The ability of CIB1 siRNA to reduce the agonist-induced production of S1P demonstrates a biological significance of the CIB1 mediated SK1 translocation. As S1P has been shown to facilitate quite varied signalling events, it would be interesting to determine whether CIB1 is critical in mediating other signalling outcomes associated with S1P production. One obvious area of interest is the affect of CIB1 on SK1-mediated proliferation. The pro-proliferative affects of SK1 are dependent upon the membrane localisation of this enzyme (Pitson et al., 2005). Hence, it would seem plausible for this proliferative signalling to also be dependent upon CIB1. Interestingly, CIB1 has been previously shown to mediate proliferation with the knockdown of CIB1 reducing proliferation in both mouse heart and lung endothelial cells (Zayed et al., 2007). Of note, these proliferative effects of CIB1 may also, at least partially, be due to the association of this protein with p21-activated kinase 1 (PAK1). CIB1 has been shown to bind to and activate PAK1 in different cell types, with CIB1 depleted cells showing reduced PAK1 activation on adhesion to fibronectin (Leisner et al., 2005). PAK1 is thought to promote cell survival through its involvement in activation of the MAPK signalling pathway (Tang et al., 2000). Additionally, PAK1 has been shown to phosphorylate BAD (a pro-apoptotic member of the Bcl-2 family of proteins) (Schurmann et al., 2000) and activate NFκB (Frost et al., 2000), with these events also involved in cell survival signalling. Hence, it is possible that CIB1-regulated proliferation is mediated through numerous pathways, with roles for both SK1 and PAK1.

Intriguingly, Spiegel and colleagues (Maceyka et al., 2008) have identified a connection between SK1 and PAK1 in melanoma cells. In this study, *in vitro* kinase assays demonstrated that S1P, but not sphingosine, activates PAK1, while a down regulation of SK1 activity was shown to decrease PAK1 activity (Maceyka et al., 2008). In their system, FLNa, an actin-binding protein involved in cytoskeletal reorganisation, was found to interact with SK1 and was required for SK1 localisation to lamellipodia in upon stimulation with Heregulin (Hrg), where SK1, FLNa and $S1P_1$ colocalise (Maceyka et al., 2008). Interestingly, both FLNa and SK1 were found to interact with PAK1, with the hypothesis that this triumvirate of proteins (FLNa, SK1 and PAK1) co-ordinately act together to regulate lamellipodia formation and promote cell movement in melanoma cells (Maceyka et al., 2008). This leads the intriguing possibility that a similar system may operate in other cells with the possibility of a CIB1-SK1-PAK1 complex. CIB1 could act to drive this

complex to the plasma membrane upon agonist stimulation, where a localised production of S1P could then act in a feed-forward manner to further activate PAK1. Hence, the proliferative effects of CIB1 may be due to concerted signalling events by both SK1 and PAK1. This theory is in part supported by the observation of decreased PAK1 activation in CIB1 depleted cells (Zayed et al., 2007).

SK1 activity is also critical in the up-regulation of the adhesion molecules E-selectin, vascular cell adhesion molecule-1 (VCAM) and intercellular adhesion molecule-1 (ICAM) on endothelial cells following TNF-α stimulation (Xia et al., 1998). The regulated expression of these adhesion molecules on the vascular endothelium following activation by cytokines is essential in the recruitment of red blood cells during the immune response (Golias et al., 2007). SK1 activity and S1P production are increased in endothelial cells following stimulation with TNF-α (Xia et al., 1998). As the up-regulation of the above mentioned adhesion molecules is inhibited by the use of DMS, but can be rescued by addition of exogenous S1P, it is thought that the role of SK1 in this process is mediated through the production of S1P and the downstream effects of this signalling molecule (Xia et al., 1998). As I have demonstrated a requirement for CIB1 in the agonist-induced production of S1P in HeLa cells, it would be interesting to determine whether this role is conserved in the vascular endothelium, and whether a role for CIB1 exists in the up-regulation of adhesion molecules following cytokine stimulation. This would potentially define a role for CIB1 in the immune response.

Interestingly, both CIB1 and SK1 appear to have roles in angiogenesis. Not only does extracellular S1P exert mitogenic activity through its activation of the ERK1/2 pathway (Kimura et al., 2000), activation of PI3K/Akt signalling (Morales-Ruiz et al., 2001) and the stimulation of endothelial cell remodelling, but a recent study has shown small, chronic increases in SK1 activity in endothelial cells enhances their ability to undergo angiogenesis in a tube formation assay (Limaye et al., 2009). This demonstrates a direct role for intrinsic SK1 activity in angiogenesis. Similarly, the role of CIB1 in angiogenesis was demonstrated through the use of endothelial cells depleted of CIB1 protein either through isolation from a CIB1 deficient mouse or generated by short hairpin RNA transfection (Yuan et al., 2006a). The loss of CIB1 in these cells resulted in reduced migration, proliferation and tubule formation (Zayed et al., 2007). In addition, CIB1 deficient mice display reduced growth-factor induced microvessel sprouting in *ex vivo* organ cultures and *in vivo* Matrigel plugs

(Zayed et al., 2007). Again, these defects in angiogenesis may be mediated through the association of CIB1 with PAK1, as hypothesised by these authors. However, it would be interesting to determine whether the angiogenic defects in the CIB1 deficient endothelial cells could be rescued by addition of exogenous S1P or expression of a SK1 variant localised constitutively at the plasma membrane (Pitson et al., 2005). Positive outcomes in these studies would suggest the role of CIB1 in vessel formation to be at least partially mediated through the SK1 pathway.

7.4 CIB2 Acts as an Endogenous Inhibitor of SK1 Translocation

CIB2 shares 59% amino-acid similarity with CIB1, and from sequence comparison, appears to be quite highly homologous with CIB1 in its structural EF-hand domains, and in the presence of a large hydrophobic binding channel (Gentry et al., 2005), where SK1 is likely to bind. Prior to embarking on the current study, very little information regarding a biological function CIB2 was known. In one recent report, CIB2 was shown to bind Ca^{2+} and interact with the integrin α7Bβ1D in skeletal muscle (Hager et al., 2008), and possibly regulate signalling through this integrin. CIB2 has also been shown to bind an αIIb cytoplasmic tail peptide *in vitro* (Denofrio et al., 2008). As CIB1 was first identified through its interaction with this integrin, it seemed likely that CIB1 and CIB2 may have similar or common functional roles in the cell.

In this study, I demonstrated the ability of CIB2 to interact with SK1 in cells, with this interaction dependent upon the same residues in SK1 critical for binding CIB1. Unlike CIB1, however, the binding of CIB2 to SK1 surprisingly occurred independent of Ca^{2+} ions. As this interaction was also independent of SK1 phosphorylation, a basal CIB2-SK1 interaction seems possible. In addition to the apparent disparities between the binding conditions regulating their respective interactions with SK1, CIB1 and CIB2 displayed differential localisations within the cell. While CIB1 utilises its myristoyl moiety to act as a Ca^{2+}-myristoyl switch, translocating from the cytosol to the plasma membrane upon mobilisation of Ca^{2+}, CIB2 showed no change in its cellular localisation upon either ionomycin or phorbol ester stimulation. This is despite CIB2 being myristoylated in these cells. The observation that CIB2 does not appear to act like a Ca^{2+} myristoyl switch, leads to questions regarding the role of its myristoylation. However, while CIB2 was largely

cytoplasmic in HeLa cells, it is possible that it may be membrane attached or targeted to membranous regions in other cells, facilitating alternate functions. The lack of a membrane localisation of CIB2 also suggests a conformation of this protein in which its myristoyl moiety is sequestered into a hydrophobic pocket, protecting it from the cellular environment.

Adding to these localisation differences, CIB1 and CIB2 appear to function in quite contrasting roles in relation to their regulation of SK1 signalling. While CIB1 was shown to mediate the agonist-induced translocation of SK1 to the plasma membrane and be important for the downstream S1P production and anti-apoptotic signalling associated with this event, CIB2 acted in a negative fashion to inhibit SK1 translocation upon agonist stimulation. Upon agonist stimulation and SK1 activation, cytosolic Ca^{2+} fluxes have been observed to occur in the cell (Spiegel and Milstien, 2003b). As CIB2 appears to interact with SK1 in a constitutive manner, independent of both Ca^{2+} ions and phosphorylation, and does not alter its cytosolic localisation upon mobilisation of Ca^{2+} stores, CIB2 may exert its inhibitory effects by retaining SK1 in the cytoplasm, despite upstream signalling events. Similar to the depletion of CIB1 protein, and overexpression of the non-myristoylated CIB1, expression of CIB2 also blocked TNF-α-associated anti-apoptotic signalling, and was able to inhibit oncogenic Ras-induced neoplastic transformation. It is likely that the downstream events, facilitated by SK1 translocation and blocked by the expression of CIB2 are a result of a loss in S1P production following agonist stimulation. To determine whether this is the case, further studies need to be completed to firstly ensure that ERK1/2 and SK1 are both phosphorylated and activated following agonist stimulation in CIB2 expressing cells, and secondly, to measure S1P levels in these conditions.

Given the common residues in SK1 required for binding to both CIB1 and CIB2, it is likely that these two proteins compete for binding to this enzyme. These interactions are most likely governed both by binding affinities and relative cellular expression levels of CIB1 and CIB2. It is possible that a basal interaction between CIB2 and SK1 is out-competed by a higher-affinity interaction with CIB1 upon mobilisation of Ca^{2+} stores, resulting in CIB1 translocating SK1 to the plasma membrane and a pro-proliferative, anti-apoptotic outcome. In some situations, however, an upregulation of CIB2 expression may occur and prevent this signalling event (Figure 7.3). To further clarify this model, surface plasmon resonance (Biacore™) studies should be undertaken to determine the relative affinities of both apo-

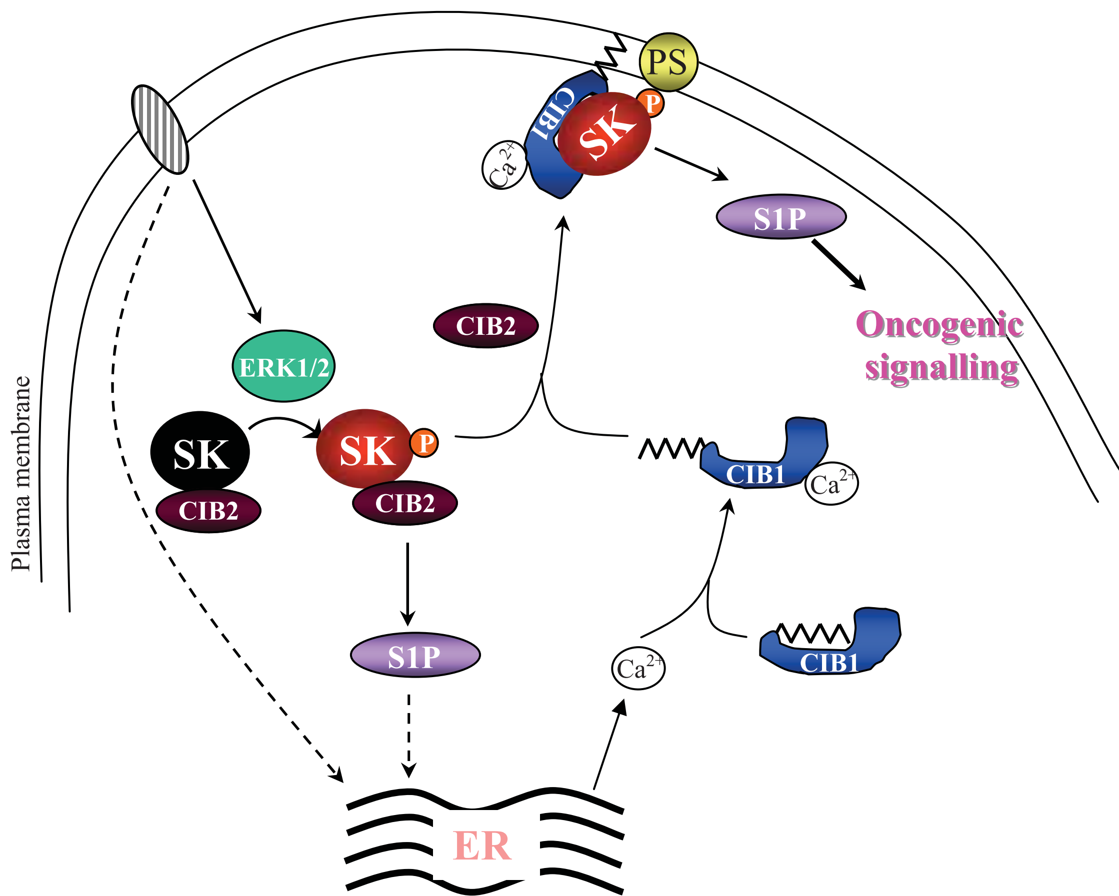

Figure 7.3. Proposed model for the concerted regulation of SK1 translocation by CIB1 and CIB2

CIB2 likely interacts with non-phosphorylated SK1 under basal cellular conditions. Following agonist stimulation, ERK1/2 phosphorylates SK1 at Ser^{225}, activating the enzyme, resulting in elevated cytoplasmic S1P and an increase in cellular Ca^{2+} proposed through release of this ion from the ER. Ca^{2+}/CIB1 may now outcompete CIB2 for binding to phosphorylated SK1 through a higher affinity association with this enzyme, and translocate SK1 to the plasma membrane through its Ca^{2+}-myristoyl switch mechanism. Once at the plasma membrane, retention of phosphorylated SK1 at this site may be mediated via its interaction with plasma membrane-associated phosphatidylserine (*PS*). SK1 at the plasma membrane generates a localized pool of S1P, which leads to pro-survival, pro-proliferative and oncogenic signaling. In some instances, however, cells may upregulate CIB2 gene expression, such that CIB1 can no longer outcompete CIB2 for binding to SK1, preventing this translocation event.

and Ca^{2+} bound CIB1 and CIB2 for SK1. In addition, quantitative RT-PCR, demonstrating the relative mRNA levels of CIB1 and CIB2 in both normal and cancerous cell lines may shed some light onto the regulation of this pathway. Interestingly, examination of microarray data from several gene expression studies supports the hypothesis that this pathway may be at least partially regulated through the relative expressions of CIB1 and CIB2. In comparison to matched normal tissue samples, several cancer types show a concurrent up regulation of CIB1 and down regulation of CIB2. This is most apparent in ductal breast carcinoma where both CIB1 overexpression and CIB2 underexpression is evident in the same cancerous tissues (Sorlie et al., 2001; Sorlie et al., 2003; Perou et al., 2000; Karnoub et al., 2007). This phenomenon is also apparent in one case of cervical cancer (Pyeon et al., 2007). Hence, these studies support the notion that the opposing roles of CIB1 and CIB2 in relation to their proliferative and survival signalling outcomes are likely regulated through relative expression levels, with a deregulation of both of these proteins (up-regulation of CIB1 and down-regulation of CIB2) leading to an oncogenic outcome.

The results from this study suggest that expression of CIB2 can negatively modulate the biological signalling outcomes associated with SK1 translocation. Hence, the expression of CIB2 likely represents a physiologic mechanism to regulate cell fate through the SK1 signalling pathway.

7.5 Potential Therapeutic Applications

7.5.1 SK1 Inhibitors and Cancer

As previously described, SK1 activity has been widely implicated in a number of different diseases, with the most extensively studied being is its role in cancer. Not only does the overexpression of SK1 result in tumour formation in allograft models in mice (Xia et al., 2000), a number of studies have identified a deregulation of SK1 in naturally occurring tumours (French et al., 2003; Van, Jr. et al., 2005; Li et al., 2008; Bayerl et al., 2008; Kawamori et al., 2006; Kawamori et al., 2009), as well as both acute and chronic myeloid leukaemia (Le et al., 2005; Li et al., 2007; Sobue et al., 2008). Encouragingly, the targeting of SK1 activity has shown promising results in the treatment of cancers *in vitro* and *in vivo*

(French et al., 2006; Kohno et al., 2006; Paugh et al., 2008), with most current approaches towards targeting this enzyme largely directed at developing small molecule inhibitors of SK1 catalytic activity (Pitman and Pitson, 2010).

One potential downfall for the use of direct catalytic inhibitors of SK1 to treat diseases such as cancer may be their lack of specificity towards tumour cells. As SK1 is ubiquitously expressed and the production of S1P, catalysed by this enzyme, has many diverse signalling roles, it is likely that broad spectrum SK1 inhibitors will have additional detrimental results in the human body. Particularly concerning is the possible adverse affects that may be caused by inhibition of the basal housekeeping role of SK1. Chemical inhibition will not only target activated SK1, but also basal SK1 activity, which may lead to a build up of sphingosine, ceramide and other upstream lipids. Increased levels of these lipids will likely result in cellular apoptosis, which may lead to detrimental affects. Of note however, no adverse health affects were observed in mice treated with chemical inhibitors of SK1 (French et al., 2006). However, the treatment of these mice was only short-term (15 days), and the longer-term affects that these inhibitors may have on the human body remain unknown. Hence, there is great potential to develop inhibitors to specifically target only the oncogenic activity associated with this enzyme, while leaving the 'housekeeping' function mediated by the basal activity of this enzyme untouched.

7.5.2 Targeting SK1 Translocation as a Cancer Therapeutic

As previously described, expression of CIB1 in cells has the potential to result in neoplastic transformation, while expression of a 'dominant-negative' CIB1 and/or expression of CIB2 blocks the transforming ability of a constitutively-active Ras. These results firstly highlight the oncogenic implications associated with the translocation of SK1 to the plasma membrane, but also draw attention to the potential ability to manipulate both SK1- and Ras-induced oncogenesis through the targeting of the translocation of SK1. This, and the ability of this protein to regulate angiogenesis, makes CIB1 appear to represent a potential new target for cancer therapeutics.

One possibility to target the CIB1-mediated SK1 transformation for cancer therapy would be to develop small molecule inhibitors of the CIB1-SK1 interaction. This could potentially

target the hydrophobic binding pocket of CIB1. As the crystal structure of CIB1 has been solved, this could be achieved through *in silico* docking studies of already compilated chemical libraries, with potential inhibitors confirmed through competitive binding analyses. In considering this approach, however, it would be important to ensure the inhibitor did not display cross-reactivity with CaM, calcineurin B, or any other CIB1-like molecule with a similar hydrophobic binding channel, such as CIB2. This approach may have limitations, as the inhibitor would not only block the CIB1-SK1 interaction, but would also be likely to interrupt the ability of CIB1 to bind many of its other interacting partners. Although many of the functions of CIB1 in respect to the signalling or regulation of its numerous interacting partners in the cell remains unknown, a global CIB1 inhibitor would likely affect processes in the cell unrelated to its function with SK1. Hence, the validity of such an approach remains unknown.

Alternatively, a potentially better approach may be to target the CIB1-binding site on SK1. The lack of a crystal structure of SK1, makes docking studies impossible, however an alternate method could be a high-throughput small molecule screen to test the ability of already known compounds to inhibit the SK1-CIB1 interaction. To specifically target SK1, hits from the primary screen could undergo a secondary screen to eliminate molecules that cross react with other known interacting partners of CIB1 such as the cytoplasmic domain of αIIb. These potential inhibitors would require further dose response, cell-permeability, purity and solubility studies. Once identified, these CIB1-SK1 interaction inhibitors could be tested for their ability to block SK1 translocation and downstream signalling effects *in vitro*. If these studies showed promising results, these compounds could be used murine cancer models to determine their effect on tumourigenesis in these models.

7.6 Functions of CIB3 and CIB4 with Respect to SK1

In addition to CIB2, this study also identified the two other CIB1-related proteins CIB3 and CIB4 as SK1-binding proteins. Each of these also interacted within the CaM/ CIB1 binding site of SK1, and similar to CIB2, interacted with SK1 independent of both metal ions and the phosphorylation state of SK1. Thus, it is likely that these proteins also interact constitutively with SK1 in the cell.

Currently, there is very little known regarding these CIB1-related proteins. From primary sequence comparison, it is predicted that these CIB1 homologs share both the four EF-hand structure of CIB1 (with Ca^{2+} binding predicted in EF-3 and EF-4) (Gentry et al., 2005), in addition to the hydrophobic binding pocket of CIB1 (Denofrio et al., 2008). In its only study, CIB3 mRNA was found to be up-regulated in CIB1 knock-out platelets (Denofrio et al., 2008). As these platelets showed no defect in activation or aggregation where CIB1 appears to play a role (Naik and Naik, 2003b; Tsuboi, 2002), it was suggested that CIB3 may compensate for the loss of CIB1 by regulating signalling through the integrin αIIbβ3 (Denofrio et al., 2008). There have been no studies to date on CIB4.

Despite suggestions for compensatory roles between each of the CIB family members, in my studies, neither CIB3 nor CIB4 appeared to have completely conserved functions with either CIB1 or CIB2 in respect to their regulation of SK1 signalling. Both CIB3 and CIB4 are myristoylated in cells, however, neither CIB3 nor CIB4 appeared to act as Ca^{2+}-myristoyl switches. Hence, a conserved role with CIB1 in mediating the translocation of SK1 to the plasma membrane seemed unlikely for either of these proteins.

CIB3 did not appear to have any observable affects on the regulation of the SK1 localisation nor any of the downstream biological outcomes that I investigated. We hypothesised that the lack of any observable affects on the SK1 signalling pathway by CIB3 are likely due to the localisation of this protein. In both unstimulated and ionomycin or PMA treated cells, CIB3 appeared to be largely present within the nucleus, in rounded vesicle-like structures that did not align with the nucleolus. However, it was unclear as to what structure this staining represented. Although it is possible that the overexpression of this protein has disrupted its physiological localisation, this staining was not observed with other protein overexpression studies. To overcome this potential issue, antibodies to detect endogenous CIB3 will need to be generated.

As the localisation of CIB3 appeared to be largely nuclear, it is difficult to perceive a role for CIB3 in the regulation of cytoplasmic SK1 signalling, with this supported by the lack of any observed effects of CIB3 expression on SK1 translocation, or anti-apoptotic signalling upon agonist stimulation. It also leads us to question whether a physiological cellular interaction of CIB3 and SK1 exists, or whether this interaction was an artefact of our *in vitro* system. However, it was quite interesting that in all of our immunofluorescent studies,

and in studies by others (Johnson et al., 2002), strong nuclear localisation of endogenous SK1 has been observed. SK1 has two functional nuclear export signal sequences, and has been found to accumulate in the nucleus upon inhibition of the nuclear export reporter CRM1 (Inagaki et al., 2003). In addition, both SK1 and SK2 have been found to colocalise with $S1P_5$ in the centrosomes of mammalian cells, where they may regulate mitosis (Gillies et al., 2009). Hence, there is a possibility that CIB3 and SK1 interact in the nucleus, and regulate a function such as mitosis, separate from the known cytoplasmic/ plasma membrane signalling roles of SK1. Future studies should examine whether this interaction does in fact exist, and the physiological significance of this.

The function of CIB4 initially appeared to be very similar to CIB2 in that both proteins localised to the cytoplasm, and expression of CIB4 also blocked the agonist-induced translocation of SK1 to the plasma membrane, and increased cell susceptibility to TNF-α mediated cell death. However, in *in vitro* focus formation assays, the expression of CIB4 enabled cells to lose contact-inhibition, resulting in numerous foci, with these not susceptible to the addition of an SK inhibitor. The ability of CIB4 to block endogenous SK1 translocation and anti-apoptotic signalling, yet result in cellular transformation is quite contradictory, and does not fit with our typical model of SK1 signalling. However, as so little is known regarding the function of CIB4, it is quite likely that this protein has numerous interacting partners in the cell. Consequently, one or more of these interactions may facilitate a role for CIB4 in other signalling pathways, masking our ability to determine the biological role of the CIB4-SK1 interaction. However, the ability of CIB4 to result in focus formation is quite interesting in itself, and future studies should determine the true oncogenic nature of this protein by initially examining other *in vitro* assays for neoplastic transformation but also in allograft tumour formation studies in mice. Additionally, should CIB4 represent a true oncogene, further studies could identify CIB4 interacting proteins through, for example, a yeast two-hybrid approach, in an attempt to identify the mechanism by which CIB4 exerts its oncogenic effects.

7.7 SK1, $S1P_2$ and Platelet Activation

Platelet activation, aggregation and subsequent clot formation are critical process in wound healing and the maintenance of vascular integrity. Platelet activation occurs through

exposure to one of a number of soluble, secreted agents, inducing a series of signalling events which enable the platelets to bind fibrinogen, link together and ultimately result in clot formation. Along with red blood cells and the vascular endothelium, platelets are also thought to be one of the largest contributors to the plasma S1P levels (reviewed in Yatomi, 2008). Platelets store large amounts of S1P, which is released upon platelet activation (Yatomi et al., 1997), and then acts to further activate neighbouring platelets, in a feed forward manner (Yatomi, 2008). Although this role for S1P in platelet aggregation has been established, the function of SK1 in platelet activation is still contentious. In addition, the intracellular signalling mechanism underlying effects of S1P in platelets is not clearly characterised. However, this may soon be rectified with a recent study identifying expression of the S1P receptor, $S1P_2$ on human platelets (Randriamboavonjy et al., 2009).

In this study, I demonstrated a critical role for SK in low-dose collagen-induced platelet aggregation. Platelets in which SK was inhibited, however still had an intact inside-out signalling pathway, consistent with previous findings (Ohmori et al., 2005). The collagen-specific aggregation defect in SK inhibited platelets is likely due to the varied signalling pathways activated by the various platelet agonists, with most platelet agonists activating PLCβ through G_q, (rather than PLCγ2 by collagen) (Ohmori et al., 2005).

Surprisingly, although SK inhibition induced no defect in shape change, nor aggregation induced by ADP, these platelets had a reduced ability to bind fibrinogen. These results are contradictory and conflict prior studies where SK inhibition had no affect on ADP-induced Ca^{2+} mobilisation (Ohmori et al., 2005; Yang et al., 1999). Although it is unclear how fibrinogen binding could be affected in the absence of an aggregation defect, the fibrinogen binding assay may be a more sensitive measurement of platelet activation. While SK inhibition may induce a defect in the ability of ADP-activated platelets to bind fibrinogen as observed in this study, this defect may be minor in terms of platelet function and not affect their ability to aggregate.

The mechanism by which SK1 appears to regulate the collagen-induced platelet aggregation is currently not clear, especially as numerous studies have observed no overall change in SK1 activity following platelet stimulation (Yatomi et al., 1997; Ohmori et al., 2005). However, Ohmori and colleagues (2005) observed an increase in SK1 activity in the membrane fraction of platelets when specifically activated through the collagen receptor.

Hence, this collagen-specific relocalisation of SK1 may generate a pool of S1P close to the platelet membrane, with this event critical in collagen-mediated platelet aggregation. As S1P released from activated platelets is predicted to result in a feed-forward activation of neighbouring platelets (Yatomi, 2008), we could speculate that the inhibition of this localized pool of S1P may be the source of the defective aggregation. This model would also explain the intact collagen-induced shape change and fibrinogen binding despite SK inhibition, observed in this study. Future studies should examine the affect of SK inhibition on S1P release from collagen-activated platelets, and whether addition of exogenous S1P could rescue the aggregation defect in these platelets.

Similar to that caused by SK inhibition, $S1P_2$ antagonism specifically inhibited low-dose collagen-induced platelet aggregation, but did not affect platelet shape change nor fibrinogen binding, suggesting that the $S1P_2$ receptor functions downstream of these events in collagen-induced platelet activation. These results are consistent with a role for $S1P_2$ in the feed-forward activation of neighbouring platelets upon exposure to secreted S1P.

As $S1P_2$ antagonism caused no overt defect in platelet function upon activation with agonists such as ADP or TRAP, we can hypothesise that these activators may utilise alternate methods of feed-forward platelet activation. For example, ADP and thrombin activation of platelets may induce a preferential release of ADP, while collagen stimulation may favour release of S1P to activate neighbouring platelets. This model is supported by the observed collagen-specific increase in SK1 activity in the membrane fraction of platelets, likely to produce a localised pool of S1P to be released (Ohmori et al., 2005). Future studies could examine whether variations exist in the levels of S1P released upon platelet activation with different agonists. This may further clarify both the role of SK1 and S1P in platelet activation and aggregation.

7.7.1 Do SK1 and CIB1 Together Regulate Platelet Aggregation?

An interesting link in this system may be between SK1 in its role in collagen-induced platelet activation, its hypothesised relocalisation to the platelet membrane, and CIB1, the protein responsible for this enzymes translocation in mammalian cells (Chapter 3). CIB1 is expressed in human platelets and hypothesized to be involved in platelet activation through

its association with the integrin αIIbβ3. Although conflicting results exist, CIB1 has been shown to activate αIIbβ3 both *in vitro* and in cells, and its direct association with αIIb increased the affinity of this integrin for fibrinogen (Tsuboi, 2002). In addition, the association of CIB1 with αIIbβ3 has been shown to be critical for proper platelet spreading on immobilised fibrinogen (Naik and Naik, 2003b). Although CIB1 can interact with αIIbβ3 regardless of its activation state, it appears to preferentially interact with active αIIbβ3, with this association necessary for the release of ADP from platelet granules (Naik and Naik, 2003a).

CIB1 has been shown to recruit the actin polymeriser WASP to αIIbβ3 post integrin activation and enhance adhesion (Tsuboi et al., 2006), suggesting a role for CIB1 in the regulation of actin cytoskeletal changes and platelet spreading during inside-out signalling. Hence, CIB1 has a clear ability to actively recruit partner proteins to the plasma membrane through its association with active αIIbβ3. It is unclear whether the Ca^{2+} -myristoyl switch function of CIB1 is conserved in platelets, however Ca^{2+} mobilisation upon platelet activation is a well-established event. Should CIB1 function in this manner in platelets, this may provide a mechanism for CIB1 to firstly interact preferentially with active αIIbβ3 upon platelet stimulation, but also to recruit other proteins involved in platelet signalling to the platelet membrane.

Due to our previous evidence of a CIB1-regulated translocation of SK1 to the plasma membrane in other mammalian cells (Chapter 3), and the potential for a relocalisation of this enzyme in platelet activation (Ohmori et al., 2005), it is tempting to speculate a role for CIB1 in the collagen-specific recruitment of SK1 to the plasma membrane upon integrin (and platelet) activation. In addition to regulating signalling through active αIIbβ3, this role of CIB1 could function to generate a localized pool of S1P at this site. This may be critical for platelet aggregation, but not required for initial platelet shape-change or fibrinogen binding, as implied by this study. Further studies examining the role of CIB1 in the plasma membrane localization of SK1 in collagen-stimulated platelets may shed further light on this potential link.

7.8 Concluding Remarks

The agonist-induced translocation of sphingosine kinase 1 to the plasma membrane is critical in mediating the pro-proliferative, anti-apoptotic and oncogenic effects of this enzyme (Pitson et al., 2005). Hence, this event represents a promising target for cancer therapeutics. In this study, I have identified the Ca^{2+}-binding protein CIB1 as mediating the rapid, agonist induced translocation of SK1 to the plasma membrane. Correspondingly, the targeting of CIB1 attenuates S1P generation, blocks the cell survival effects of SK1, and inhibits oncogenic Ras transformation. These regulative effects of CIB1 make it a potential target in cancer therapeutics. As CIB1 has multiple binding partners in the cell, and likely regulates other cell signalling events (reviewed in Yamniuk and Vogel, 2006), the most attractive possibility would be to inhibit the SK1-CIB1 interaction. In theory, such an inhibitor could disrupt the translocation of SK1, blocking the associated downstream oncogenic signalling, without affecting either the basal activity of SK1 in the sphingomyelin cycle, nor the ability of CIB1 to regulate its various other targets within the cell. Hence, further research into the oncogenic potential of CIB1 and a potential inhibitor for its interaction with SK1 may be useful for future cancer therapeutics.

This study also identified the closely related-CIB1 protein, CIB2 as a natural inhibitor of SK1 translocation. Expression of CIB2 was also able to block the cell survival affects of SK1 and inhibit Ras transformation, showing the ability of this protein to block downstream biological outcomes associated with SK1 membrane localisation. Hence, it appears that CIB2 may represent a physiologic mechanism to regulate the translocation of SK1 to the plasma membrane, and hence, the downstream signalling outcomes facilitated by this. As it is likely that CIB1 and CIB2 compete for binding to SK1, further research into the regulation of these interactions, to determine the relative binding affinities for SK1 and the expression levels of each of these proteins in both normal and cancerous cells may shed further light onto the regulation of SK1 translocation in both normal and disease states.

The two other CIB1-related proteins CIB3 and CIB4 were also identified as SK1-interactors, however, their roles in respect to SK1 remain ambiguous following this study. While suggestions of compensation between the CIB proteins have been proposed, in this study, neither CIB3 nor CIB4 appeared to completely conserve a role with either CIB1 or CIB2 in their opposing functions in the regulation of SK1 translocation. The nuclear

localisation of CIB3 makes it difficult to perceive a role in the regulation of cytoplasmic SK1, and this was supported by the lack of any observable affects on the SK1 signalling pathway upon CIB3 expression. While it initially appeared CIB4 may block SK1 translocation and anti-apoptotic signalling, preliminary studies suggest this protein may also be oncogenic, independent of the SK1 pathway. Future studies into additional binding partners of CIB4 may shed further light onto this contradictory behaviour.

Finally, this study has identified a role for SK1 and the S1P receptor $S1P_2$ in collagen-induced platelet aggregation. SK1 and $S1P_2$ appear critical after the initial inside-out signalling event and fibrinogen binding to activated platelets but prior to aggregate formation. Hence, this role for SK1 may function in an S1P-dependent feed-forward activation of neighbouring platelets. Further experimentation to identify the exact signalling mechanisms of SK1 in platelets may further our understanding of the role of this enzyme in blood clotting.