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

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Thesis amendments

Examiner 1

Discussion on the limitations of using genetic manipulations of CIB1 to study the function of SK1.

Added to Section 7.2 (Page 96)

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 in the cell. 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.

Examiner 2

Figure 3.1 has been amended to include an arrow showing endogenous SK1.

Section 3.3.1 (page 46) has been amended to include comments on both the anti-SK1 and anti-CIB1 antibodies. It now reads:

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.

In Materials and Methods, Section 2.11 has been amended slightly to now read:

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.

In each of Figures 3.3, 3.5, 3.6, 3.8 and 3.11, the number of cells used for membrane quantitation is now indicated in the figure legends.

In relation to membrane ruffles, the following paragraph has now been added to the discussion in Chapter 3 (page 55):

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.

Chapter 5.3 (page 72) has been amended to now read as following:

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.

Typographical Errors:

- Page 2a: 'degradaded' has been changed to 'degraded'.
- Page 3: 'golgi' now has a capital 'G'.
- Page 7: 'phosphatidyl inositol is now written as one word.
- Page 8a: 'parocrine' is now amended to 'paracrine'.
- Page 9: 'triphosphate' is now corrected to 'trisphosphate'.
- Page 9a: 'triphosphate' is now corrected to 'trisphosphate'.
- Page 10: 'epinephrine' has been replaced with 'adrenaline'.
- Page 13: 'BCR/ABL fusion' has been replaced with 'BCR/ABL gene fusion'.
- Page 43: 'Prevent' has been changed to 'prevented'.
- Page 50: 'Translocate' has been changed to 'translocated'.
- Page 80: 'Dependant' has been changed to 'dependent'
- Page 91: 'Dependant' has been changed to 'dependent'.
- Page 92: 'Dependant' has been changed to 'dependent'.

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Abbreviations

ABC;	ATP Binding Cassette
AC;	Adenylate Cyclase
ACD;	Acid Citrate Dextrose
ADP;	Adenosine diphosphate
ATP;	Adenosine triphosphate
AML;	Acute myeloid leukaemia
BAL;	Broncho-alveolar lavage
BAPTA/AM;	1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester
BSA;	Bovine serum albumin
CaM;	Calmodulin
Cdc42;	Cell Division Cycle 42 (GTP binding protein)
CHX;	Cycloheximide
CIB;	Calcium-and-integrin binding protein
CML;	Chronic myeloid leukaemia
CRP;	Collagen-related peptide
DNA-$PK_{CS;}$	DNA-dependent protein kinase
DMS;	*N,N*-dimethylsphingosine
DTT;	Dithiothreitol
EGF;	Epithelial growth factor
ER;	Endoplasmic reticulum
ERK1/2;	Extracellular Signal Regulated Kinase 1/ 2
ESTs;	Expressed sequence tags
FAK;	Focal Adhesion Kinase
FLNa;	Filamin A
GFP;	Green Fluorescent Protein
GPVI;	Plycoprotein VI
GSH;	Glutathione-sepharose
GST;	Glutathione *S*-transferase
HA;	Hemaglutanin
HDL;	High density lipoproteins
HEK293;	Human Embryonic Kidney 293 cells
Hrg;	Heregulin
HRP;	Horseradish Peroxidase
ICAM;	Intercellular Adhesion Molecule
ICRAC;	Intracellular Calcium Release-activated Calcium Current
$InsP_{3;}$	Inositol 1,4,5-triphosphate
IPTG;	Isopropyl 1-thio-β-D-galactopyranoside
JNK;	Jun N-terminal Kinase
KIP;	Kinase Interacting Protein
LDL;	Low density lipoproteins
LPA;	Lysophosphatidic acid
LPP;	Lipid Phosphate Phosphatase
MAPK;	Mitogen-activated Protein Kinase
MEFs;	Mouse embryonic fibroblasts
NBR1;	Next to BRCA1
NCS;	Neuronal calcium sensor

NGF;	Nerve Growth Factor
NMR;	Nuclear magnetic resonance
OG-FNG;	Oregon-green fibrinogen
OVA;	Ovalbumin
PA;	Phosphatidic acid
PBS;	Phosphate-buffered saline
PAK;	P21-activated kinase
PCR;	Polymerase chain reaction
PDGF;	Platelet-derived Growth Factor
PI3K;	Phosphatidyl inositol-3-kinase
PLC;	Phospholipase C
PMA;	Phorbol 12-myristate 13-acetate
PPP;	Platelet poor plasma
PRP;	Platelet rich plasma
PS;	Phosphatidylserine
PS2;	Presenilin 2
PWB;	Platelet wash buffer
RT-PCR;	Reverse transcriptase PCR
TNF-α;	Tumour Necrosis Factor-α
TRAF2;	Tumour Necrosis Factor-α Receptor-associated Factor 2
TRAP;	Thrombin Receptor-activating Protein
siRNA;	Small interfering RNA
SDS;	Sodium dodecyl sulfate
SDS-PAGE;	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH3;	Src homology 3
SK1/2;	Sphingosine Kinase 1/2
S1P;	Sphingosine-1-phosphate
$S1P_{1-5}$;	Sphingosine-1-phosphate receptors 1-5
SPP1/2;	S1P phosphatases 1/ 2
VEGF;	Vascular Endothelial Growth Factor
VCAM;	Vascular Cell Adhesion Molecule
WASP;	Wiskott-Aldrich Syndrome Protein
W7;	N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride

Abstract

Sphingosine kinase 1 (SK1) catalyses the conversion of sphingosine to sphingosine-1-phosphate (S1P). Since elevated levels of cellular S1P have a well characterised pro-survival, pro-proliferative and oncogenic effect in cells, the regulation of SK1 activity is the subject of much current focus. Cells typically have low basal levels of SK1 activity, which appears to have a 'housekeeping' role in the sphingomyelin cycle. However, the catalytic activity of this enzyme can be increased by a number of growth factors, cytokines and other agonists, generating a greater pool of S1P, which then appears to be involved in its cellular signalling.

The Pitson group have previously demonstrated that the agonist-induced activation of human SK1 is mediated through phosphorylation of this enzyme at Ser^{225}. Following this phosphorylation, SK1 translocates from the cytosol to the plasma membrane, with both of these events critical for the pro-survival, anti-apoptotic and oncogenic effects of this enzyme. Prior to the current study, the mechanism for the rapid, agonist-induced translocation of SK1 to the plasma membrane remained undetermined. Previous studies have demonstrated the requirement of the calmodulin binding site in SK1 for this translocation event, however a direct requirement for calmodulin itself has yet to be shown.

In this study, we show that calcium- and integrin-binding protein 1 (CIB1), a calmodulin-like molecule, mediates the agonist-induced translocation of SK1 to the plasma membrane. We also demonstrate the ability of CIB1 to act as calcium-myristoyl switch, providing a functional mechanism by which it can mediate SK1 localisation to the plasma membrane. In addition, CIB1 was shown to be critical for the agonist-induced production of S1P and also the anti-apoptotic signalling associated with SK1. Furthermore, CIB1 itself was shown to be potentially oncogenic, and a dominant-negative version of CIB1 was also able to inhibit H-Ras-induced oncogenesis.

These studies have also investigated the three other members of the CIB family of proteins, CIB2, CIB3 and CIB4 and their roles in regulating SK1 localisation and subsequent signalling events. While CIB1 was critical for the agonist-induced translocation of SK1 to the plasma membrane, CIB2 acted in an opposite manner, blocking the translocation of this

enzyme. Furthermore, expression of CIB2 enhanced cellular apoptosis, presumably through its inhibitory effects on the SK1 survival pathway. In addition, CIB2 blocked H-Ras mediated oncogenesis. Hence, CIB1 and CIB2 appear to have opposing roles in the cell in relation to the regulation of SK1 signalling. CIB3 and CIB4 also appeared to interact with SK1 *in vitro*, however the cellular function of these interactions was not elucidated in this study. Interestingly, despite having considerable sequence similarity to CIB1 and CIB2, neither protein acted in a similar manner to either CIB1 or CIB2 with respect to SK1 function. While CIB3 did not appear to have any role in the SK1 signalling pathway, studies suggested CIB4 likely to have additional binding partners in the cell masking the ability to determine the biological function of the CIB4-SK1 interaction.

Overall, this study identified both the translocation mechanism of SK1, in addition to a natural suppressor of this event. As the localisation of SK1 at the plasma membrane is critical in the oncogenic signalling of this enzyme, these findings may represent potential new directions for anti-cancer therapeutics.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Kate Elizabeth Jarman and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Kate Elizabeth Jarman

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