The Relevance of the SIRP Protein Family to Signal Transduction and Cell Adhesion

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Erklärung

Hiermit erkläre ich, daß ich die vorliegende Arbeit selbstständig verfaßt und keine anderen als die von mir angegebenen Hilfsmittel benutzt habe.

Ferner erkläre ich, dass ich weder an der Universität München noch an einer anderen Universität versucht habe, eine Dissertation einzureichen.

München, Juli 2001

Charles Cant

For my Mother and Father

and to Susanne

1 Introduction

The Signal Regulatory Proteins (SIRPs) are a family of transmembrane glycoproteins that recently entered the field of signal transduction. One subtype of the SIRP family, SIRP α, exerts a negative influence on signal transduction through receptor tyrosine kinases (RTK) by its association with certain phosphotyrosine phosphatases and possibly tyrosine kinases[1]. This negative regulation may have a tumour-suppressive effect and is therefore of considerable medical interest. It has come to light that SIRPs constitute a family that is in turn a member of the Inhibitory Receptor Superfamily, which has been primarily studied from an immunological perspective. Since SIRP family members are also expressed in many non-haematopoietic cell types, they take the concept of inhibitory receptors out of the immune system and into the realm of general signal transduction. For reviews on existing inhibitory receptor superfamily members and mechanisms, see [2-9]. While SIRP family members are certainly related to a number of immune receptor families, they are also analogous to members of the ubiquitously-expressed carcinoembryonic antigen (CEA) superfamily. The CEA superfamily includes the biliary glycoproteins (BGP) and the C-CAM family of cell surface adhesion molecules. Despite sharing structural homology with these families, SIRPs have not yet been shown to have the capacities to act as adhesion molecules. For a current review concerning the CEA superfamily, see Hammarstrom (1999)[117].

1.1 Structural characteristics of the SIRP α and β subtypes

The SIRP family currently numbers more than 15 closely related members among which there exists variation in the form of subtle amino acid differences in their extracellular domains. The conservation is extremely high between different family members of the same subtype and in the extracellular domain the sequences of the alpha and beta subtypes is almost as highly conserved. The family can additionally be divided into two structurally distinct subtypes designated SIRP α and SIRP β that differ by the presence or absence of an intracellular domain, respectively. Figure 1 depicts a protein sequence comparison of two members of

the SIRP α subtype, SIRP α1 and SIRP α2, and one member of the SIRP β subtype, SIRP β1.

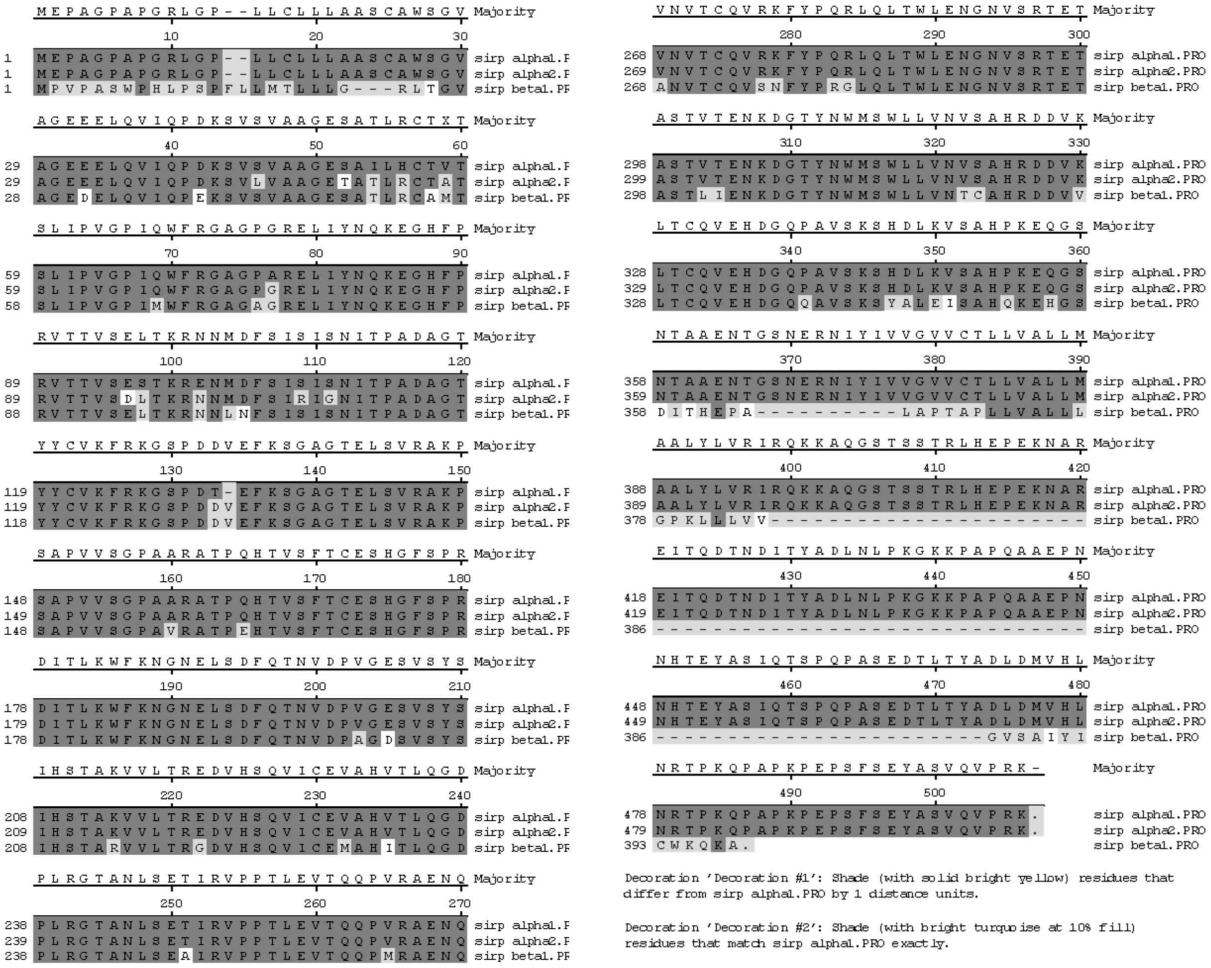


Figure 1, Amino acid comparison of the primary protein sequences of SIRP α1, SIRP α2, and SIRP β1. Identically matching protein sequence is shown in dark grey.

The SIRP α subtype has an apparent molecular weight of between 85 and 90 kDa in humans and rats and 110-120 kDa in the mouse. SIRP αs are extensively glycosylated and have either three or one immunoglobulin (Ig) -like domains. A single transmembrane domain separates the extracellular region from a cytoplasmic domain of approximately one hundred amino acids containing four

tyrosines, three of which conform to the current 'immunoreceptor tyrosine-based inhibitory motif' (ITIM) consensus, 'V/I/L/SxYxxL/V/I/S'[10]. ITIMs are present in a large group of molecules that negatively regulate cell functions. Treatment of cells with a variety of receptor stimuli phosphorylates the tyrosines in the ITIM and provides a binding site for SH2 domains often belonging to a tyrosine phosphatase (see later). A proline-rich region near the C-terminus of SIRP α may represent a binding site for SH3 domain-containing molecules [1,11]. SHPS-1, p84, BIT, MFR and MyD-1 are all SIRP α family members that have been discovered by other groups [1,12-17]. For simplicity, all these molecules shall be referred to here as SIRP α.

The shorter subtype, SIRP β, is more than 90% identical to SIRP α in its extracellular domain excluding the signal peptide and has an apparent molecular weight of 50-55 kDa. However, SIRP β possesses a dissimilar transmembrane domain with a positively charged lysine residue and has no significant intracellular region.

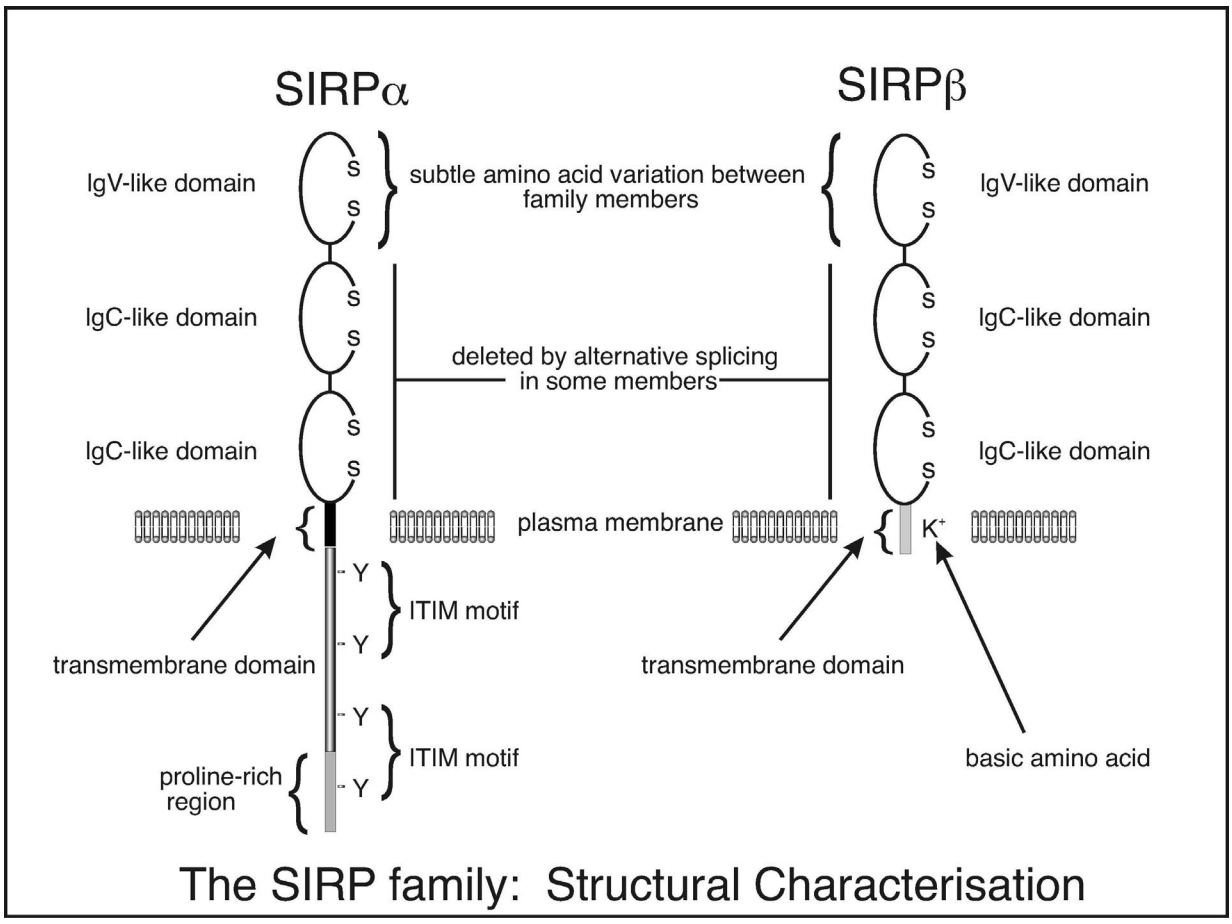


Figure 2 Structural characterisation of the SIRP family. The SIRP family consists of two structural subtypes that differ by the presence or absence of an intracellular domain. The SIRP α subtype possesses a long cytoplasmic domain with a proline-rich region near the C-terminus and two ITIM motifs that can bind SH2 domain-containing phosphatases. SIRP β has an unusual transmembrane domain containing a charged amino acid, but has no cytoplasmic domain.

1.2 Extracellular domain – structure and ligands

The extracellular domain of the SIRPs can have three Ig-like domains. The N-terminal Ig-like domain is of the Ig V-type and the majority of the sequence variation between different members of the same subtype lies in this region. The second and third domains are of the Ig C1 type and are encoded by an exon that may be removed by alternative splicing[1,12,15,18,19]. Ig-like domains usually indicate the extracellular association between one or more ligands, so it is likely that the SIRPs have binding partners outside the cell.

SIRPs are usually highly glycosylated. The putative N-glycosylation motif (NXS/T) is present 15 times in mouse [15], five times in human SIRP α and 6 times in human SIRP β. One likely reason for the difference in size seen in SDS-PAGE between mature full-length SIRP α proteins of different species is that they are glycosylated to varying degrees. The pattern of glycosylation is probably necessary for molecular recognition (see below) and may produce altered ligand specificity between SIRP α and SIRP β.

1.3 Distribution of SIRPs among tissue and cell types

SIRP α molecules are expressed in a variety of tissue types and at different levels. Two transcripts of 4.4 and 2.4 kB have been detected by Northern hybridisation in heart, brain, placenta, lung, liver, spleen and spinal cord[19,20]. RNAse protection analysis also reveals evidence of two transcripts[20]. These two transcripts probably reflect the two known variants generated by alternative splicing.

SIRP α protein is highly expressed in myeloid cells[16,20,21]. Macrophages may constitute a useful experimental system because they express a single SIRP α far more predominantly than other members[15,20,22-24]. High levels of SIRP α protein are also seen in neurons and specific areas of the brain[13,18,19,25]. In Western blot, one study collectively detected proteins at 120, 85-95, 50 and 42 kDa in different tissues and cell lines[20]. Following enzymatic deglycosylation, only two bands at 65 and 35 kDa are detectable[1,20]. Glycosylation is therefore probably critical for determining a repertoire of roles for different family members.

Approaches using detection with monoclonal antibodies have been informative but occasionally misleading by suggesting that expression is highly restricted to certain tissues. This is probably because the antibody is sensitive to a certain glycosylation pattern[15]. SIRP α glycosylation therefore is likely to vary between cell types and may reflect potential ligand binding sites being masked or created.

For some reason, it is very difficult to detect expression of endogenous SIRP β molecules at the transcriptional level by Northern blot or at the protein level by Western blot. Despite these difficulties, SIRP β was detected in myeloid cells by Western blot and can be readily amplified from cDNA isolated from a variety of

epithelial and haematopoietic cell lines. Additionally, numerous members of the SIRP β subtype were obtained from a cDNA library screen in a multitude of cell lines (Kharitonenkov *et al.* and Sures, unpublished observation), proving their endogenous expression.

1.4 Induction of SIRP α tyrosine phosphorylation

There are a variety of different stimuli that result in tyrosine phosphorylation of SIRP α. These include EGF, PDGF, insulin, neurotrophins, G-protein coupled receptors, adhesion to fibronectin, growth hormone, colony stimulating factor and serum [1,12,22,26-33]. SIRP α tyrosine phosphorylation is also induced by light in the suprachiasmatic nucleus of the brain and may therefore be involved in modulating the circadian rhythm[34]**.** The only ligands known so far to be unable to elicit SIRP α tyrosine phosphorylation are leukaemia inhibitory factor and interferon γ, shown in fibroblasts [31]. This large list of receptors includes examples with and without kinase activity, and the mechanisms by which they may transduce a signal are often very different. This begs some kind of standardisation between the manner or mechanism by which SIRP α tyrosine phosphorylation is triggered. Here, a system is proposed that funnels the receptor stimulus through a bottleneck involving cytosolic tyrosine kinases.

Tyrosine phosphorylation of SIRP α following insulin stimulation of RAT1-IR cells occurs mainly on the last two tyrosines (Y449 and Y 473)[35]. Mutating either of these tyrosines abolishes most of the tyrosine phosphorylation of SIRP α and its association with SHP-2. Which tyrosines become phosphorylated may vary depending on the nature of the stimulation.

1.5 Association of SIRP α with tyrosine phosphatases

Possibly the best characterised effect of SIRP α tyrosine phosphorylation is its strong association with either of the two SH2 domain-containing tyrosine phosphatases SHP-1 and SHP-2[1,20,22] which is mediated by the SH2 domains of the phosphatase and the phosphorylated ITIMs of SIRP α[35]. Binding stimulates the catalytic activity of the phosphatase while, perhaps paradoxically, SIRP α

molecules are also substrates of these phosphatases[1,22,27,29,35]. Structural data following the crystallisation of SHP-2 reveals that the binding of the N-terminal SH-2 domain regulates the phosphatase activity while the C-terminal domain plays an accessory role[36]. Although two molecules of SHP-2 can bind *in vitro* to one molecule of tyrosine phosphorylated SIRP α[14],a binding (which would saturate the tyrosines), other SH2 domain-containing proteins such as Grb2 and CSK can also bind to SIRP α[1,20]. A situation where proteins are heterogeneously clustered around SIRP α would make a mechanistic signalling complex easier to conceive.

Despite having a similar structure, SHP-1 and SHP-2 appear to possess different biological functions, SHP-1 having an inhibitory role and SHP-2 seemingly the opposite (reviewed in[37,38]). One function of tyrosine phosphorylated SIRP α in haematopoietic cells may be to direct SHP-1 to a receptor e.g. the CSF-1R[22], where it dephosphorylates target members of the complex. An explanation for SHP-2 association is less clear. Recruitment to SIRP α from the cytosol to the membrane following stimulation[28,35] may sequester SHP-2 away from areas where it has a positive effect. SHP-2 could activate proteins by dephosphorylation of src-family kinases as has been recently suggested[39] because the SH3 domain of c-src associates with SHP-2 that dephosphorylates Y527 *in vitro*[40]. The SH3 domain binding explains why overexpression of SHP-2 causes an increase in c-src activity that is independent of SHP-2 catalytic activity, dephosphorylation of Y527 not being observed[41]. This may have been different if the system had involved SIRP α binding to activate SHP-2.

SIRP α may rely on other proteins for negative signalling that associate in a tyrosine phosphorylation-dependent manner (see later). Dephosphorylation of SIRP α by SHP-2 would dismantle such a negative signalling complex.

1.6 Involvement of protein tyrosine kinases in SIRP α signalling

Endeavours have been made to identify the kinase or kinases responsible for direct tyrosine phosphorylation of SIRP α. Tyrosine phosphorylation of SIRP α occurs as early as one minute after stimulation of cells with EGF or PDGF[1].

Additionally, purified EGFR[28] and semi-purified IR[27] can phosphorylate SIRP α *in vitro*. Therefore, following EGF, PDGF or insulin stimulation of cells, the respective receptors are either directly responsible for tyrosine phosphorylation, or the kinase responsible is an intimate part of the complex.

However, SIRP α is also tyrosine phosphorylated after stimulation of receptors without tyrosine kinase activity. Stimulation of integrins by adhesion of cells to fibronectin leads to SIRP α tyrosine phosphorylation after 15 minutes [33,39]. This tyrosine phosphorylation is reduced when cell lines where c-src, fyn or FAK are not expressed and in cell lines overexpressing CSK, a tyrosine kinase that inhibits src family kinases by phosphorylating Y527, a regulatory tyrosine present at the C-terminus (for reviews, see [42,43]). C-src but not FAK, phosphorylates SIRP α *in vitro*[33]. Similar effects on SIRP α tyrosine phosphorylation are seen with lysophosphatidic acid (LPA), which stimulates cells through G protein-coupled receptors (GPCRs) [30]. It is important to note that examples of ligand-independent RTK stimulation following activation of integrins or GPCRs are both documented [44,45], therefore SIRP α could be phosphorylated following RTK activation by a form of lateral signalling.

Stimulation of cells with growth hormone (GH) leads to SIRP α tyrosine phosphorylation. The GH receptor has no intrinsic tyrosine kinase activity and signalling through the GH receptor is expected to be mediated by the cytosolic tyrosine kinase JAK2. Stofega et. al[46] propose that JAK2 phosphorylates SIRP α since it is able to *in vitro.* Furthermore, JAK2 is present in a complex with SIRP α when both JAK2 and SIRP α are overexpressed, an association, which requires neither the C-terminally, located proline-rich region of SIRP α nor SIRP α tyrosine phosphorylation[46]**.** Moreover, both JAK2 and SIRP α associate directly with SHP-2[31]. Lastly, overexpression of SIRP α negatively regulates GH-induced phosphorylation of MAP kinase and STAT family members.

Two further cytosolic tyrosine kinases are relevant to SIRP α. CSK associates endogenously with SIRP α [20] in macrophages but the physiological significance is unclear. SIRP α may downregulate signalling through CSK by the inhibition of src-family PTKs.

PYK2, a cytosolic tyrosine kinase related to FAK, was recently shown to be present in an *in vivo* complex with SIRP α in mouse macrophages. PYK2 represented a significant proportion of the kinase activity in this complex[23]. Whether or not CSK and PYK2 are relevant to SIRP α tyrosine phosphorylation, they are likely to be participants in SIRP-related signalling.

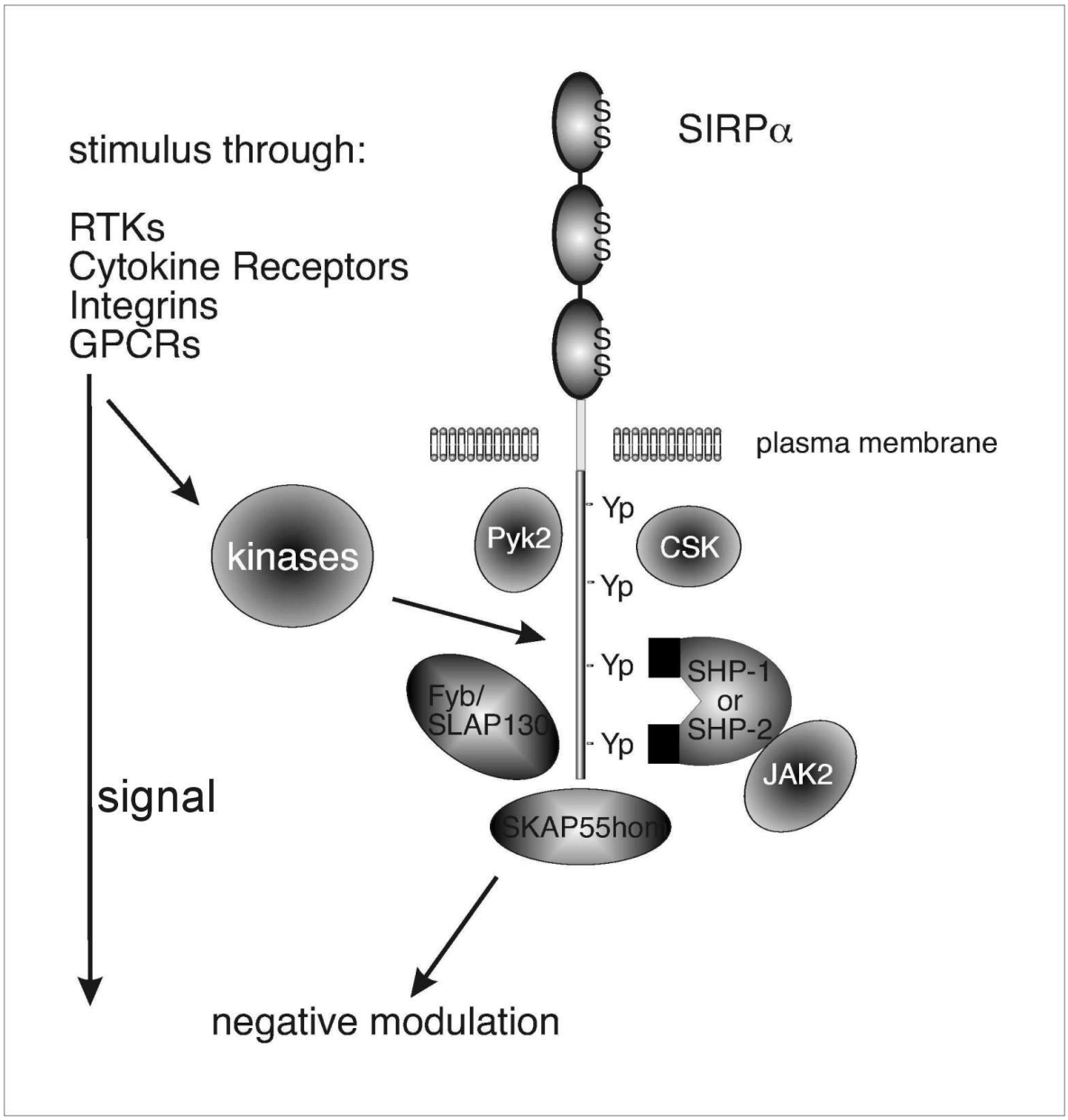


Figure 3 The SIRP α subtype: known interactors and its putative influence upon signal transduction. The diagram depicts proteins thought to be physically associated with either SIRP α proteins. A number of cytosolic tyrosine kinases cluster around SIRP α as well as two adaptor proteins ('Role of SIRPs in biological processes', see later).

Stimulation of a variety of receptors results in SIRP α tyrosine phosphorylation, thought to be catalysed by cytosolic tyrosine kinases. The consequent binding and activation of SHP-2 is necessary for an inhibitory effect upon the signalling generated by this stimulus.

1.7 Role of SIRPs in biological processes

NIH3T3 cells overexpressing SIRP α grow more slowly than mock infected cells and are resistant to focus formation and growth following transformation by v-fms. Furthermore, MAP kinase activation is inhibited following EGF or insulin stimulation in these cells[1]. On the other hand, a positive effect of SIRP α on MAP kinase activation has been reported following insulin stimulation[28].

SIRP α expression increases during macrophage fusion. Additionally, macrophage fusion is inhibited by the addition of mAb against SIRP α[15]. Similar experiments show that mAb against SIRP α inhibit the migration of neutrophils through endothelium[21]. In neuronal cells, SIRP α is documented as an adhesion molecule and implicated in neuronal outgrowth and axon guidance[13]. SIRP α molecules may therefore be relevant to macrophage fusion, cell migration or the navigation of neurons during development, but these findings, taken together with the wide tissue expression of SIRP α, suggest that their role is more fundamental, perhaps lying in specific intercellular recognition.

1.8 SIRP β: A counterpart of SIRP α?

Researchers have invested a considerable amount of work and time to understand SIRP α, its profile of expression, associated proteins, mechanism of action and biological function. As a consequence, much is known about the function of SIRP α as a molecule with dual influence on signal transduction and the possible coordination of cellular movements. During this intensive phase of investigation however, research on the SIRP β subtype was neglected. Very little was discovered about any signalling mechanisms involving SIRP β or any role it had in these or other cellular processes. There may be understandable reasons for this; SIRP β, unlike SIRP α, has no intracellular domain, and cannot therefore be tyrosine phosphorylated. Additionally, the subtype has an apparent MW (55kDa) in SDS PAGE that is the same as that of antibody heavy chains, making it very difficult to reveal through immunoprecipitation experiments. Taken together, one can see that SIRP β is technically a particularly difficult molecule to work with.

Nevertheless, multiple copies of cDNAs encoding for proteins of the SIRP β subtype have been found from cDNA library screens, and, although less strongly expressed, SIRP β can be amplified from cDNA obtained from cell lines and tissue (I.Sures, personal communication)[1]. The strong similarity of SIRP β to SIRP α throughout large regions of the protein sequence (Figure 1) may relate it to SIRP α mechanistically and also functionally. SIRP β may complement or oppose SIRP α in its function. Indeed, the two subtypes may constitute two halves of the same system, and in this case an understanding of SIRP β function would be essential to understand the overall contribution of the family to the biology of an organism.

The actions of the SIRP family suggests that modulation of receptor stimuli by inhibitory receptors may be a fundamental concept in mammalian signal transduction. In B cells and T cells, antigen receptor signalling is controlled by coreceptors recruiting cytosolic complexes in response to stimulation with antigen. The inhibitory receptor superfamily had been thought to be specific to the immune system but SIRPs show that this concept applies not only to antigen receptors, but also to receptor tyrosine kinases, cytokine receptors, G protein-coupled receptors, integrins and other membrane receptor systems. It seems, however, that immunology provides an increasingly valuable source of analogy to which we can look for inspiration.

1.9 Objectives

Knowledge about the SIRP family is limited to a window focussed upon the signalling of the intracellular domain of SIRP α. This work plans to expand it to encompass the extracellular region of SIRP α as well as the complete SIRP β molecule to build an understanding of the function of the whole family.

The role of SIRP α to regulate signal transduction and cellular transformation through receptor tyrosine kinase (RTK) signalling is known. Other molecules participating in the system could however be revealed by experiments with inhibitors of different signal transduction molecules. This would help to clarify how SIRP α is activated by a receptor.

The extracellular domain of the SIRPs has not been studied, although it comprises more than half of the molecule and always contains at least one Ig-like domain. In other molecules, Ig-like domains are often binding sites for ligands. Examining the extracellular domains for their cell-binding affinity would establish and identify new molecules that might associate with the extracellular domain of the two subtypes. Knowledge of these binding proteins could clarify the context of SIRP α signalling. A ligand common to both SIRP α and SIRP β, would possibly point to a common function.

SIRP α is known to be present at the cell membrane, to recruit other molecules to the membrane and to associate with receptors. To learn whether the SIRP β molecules had a similar localisation, perhaps within specific subcellular compartments, a combination of fluorescence microscopy and cellular fractionation techniques could be used.

To investigate the possible role of SIRP $\beta 1$ in signal transduction, its binding partners needed to be identified using appropriate *In vitro* association experiments. Assays of tyrosine phosphorylation and catalytic activity would then be necessary to reveal evidence of signalling by SIRP β-associated molecules. Within this context, coexpression of SIRP α might reveal an influence upon this signalling.

To identify a biological function for SIRP β, different cellular assays could be performed upon cell lines stably overexpressing parts of the SIRP β complex.

2 Abbreviations Used

α	anti-
aa	amino acid
Ab	antibody
aa	amino acid
AP	alkaline phosphatase
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
BCR	B cell receptor
cDNA	complementary DNA
CIP	calf intestine phosphatase
CMV	cytomegalovirus
Coom.	Coomassie
CSK	c-src kinase
ddH_2O	doubly distilled water
DAG	diacylglycerol
DAPI	4´,6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DAP12	DNAX activating protein 12
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
EGF	epidermal growth factor
ex	extracellular (domain)
FAK	focal adhesion kinase
PDGF	platelet-derived growth factor
GFP	Green Fluorescent Protein
GH	growth hormone
GST	glutathione-S-transferase
GPI	glycophosphotidyl inositol
GPCR	G-protein-coupled receptor
HRP	horseradish peroxidase
h	human
hr	hour
Ig	Immunoglobulin
IR	insulin receptor
IRES	internal ribosomal entry site
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
JAK	JAnus Kinase
JnK	c-jun kinase
kB	kilobase
kDa	kiloDalton
KIR	killer inhibitory receptor
IAP	integrin associated protein
IRES	Internal ribosomal entry site
LB	Luria-Bertani Medium
LPA	lysophosphatidic acid
LTR	long terminal repeats
MAPK	mitogen activated protein kinase
mAb	monoclonal antibody
2-ME	2-mercaptoethanol
MCS	multiple cloning site
MW	molecular weight
mRNA	messenger RNA
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI-3K	phosphoinositide 3-kinase
PLC	phospholipase C
PoV	sodium pervanadate
RNA	ribonucleic acid
RT	room temperature
RTPCR	reverse transcriptase PCR
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulphate
sec	second
SIRP	signal regulating protein
STAT	signal tranducer and activator of transcription
TAE	Tris acetate buffer
TBS	Tris buffered saline
TCR	T-cell receptor
TNFα	tumour necrosis factor alpha
T_m	melting temperature
Tris-Cl	Tris hydrochloride
U	unit of enzymatic activity
UV	ultraviolet
v/v	volume-per-volume
PLIC	‘proteins linking IAP to the cytoskeleton[47,]

3 Materials

3.1 Antibodies

The following primary antibodies were used either in immunoprecipitation or as primary antibodies in immunoblot or immunofluorescence analysis:

Antibody	Comments	Source
α-SIRP α ex(ExC_2)	Affinity purified antiserum recognizing the extracellular domain of SIRP α 1	Self-Made
α-SIRP β ex	Affinity purified antiserum recognizing the extracellular domain of SIRP β 1	Self-Made
α-SIRP ex (ExF_1)	Polyclonal antiserum recognising both SIRP α and SIRP β extracellular domains	Z.Chen[1]
B1D5C3	mAb selectively recognising the extracellular domain of SIRP β1 without recognising SIRP α1, used in stimulation experiments and immunofluorescence	Dr. H-J Bühring, Tübingen
4G10	anti-phosphotyrosine mAb	UBI, USA

The following HRP-conjugated secondary antibodies were used in conjunction with the ECL kit to visualize proteins in immunoblot (Western blot) analysis:

Antibody	Dilution	Source
Goat anti-Mouse	1: 20000	BioRad (Munich)
Goat anti-Mouse	1: 10000	Sigma (Munich)
Goat anti-Rabbit	1: 20000	BioRad (Munich)

Goat anti-mouse antibody coupled to the Cy3 Fluorophore (Molecular Probes, Leiden, Netherlands) was used in a 1:1000 dilution to visualize cellular immunostaining with mAb B1D5C3.

3.2 Chemicals, enzymes and commercial kits

3.2.1 Chemicals

Acrylamide	National Diagnostics, Atlanta, USA
Agar	Difco, Detroit, USA
Agarose	Gibco BRL, Eggenstein, Germany
Ammonium persulphate	Serva, Heidelberg, Germany
Ampicillin	Roche, Mannheim, Germany
Aprotinin	Sigma, Munich, Germany
ATP	Pharmacia, Freiburg, Germany
BES (N,N-Bis (2-Hydroxyl)	Sigma, Munich, Germany
2-amino-ethanesulphonic acid	Roth, Karlsruhe, Germany
Bis-acrylamide	National Diagnostics, Atlanta, USA
Bromophenol blue	Sigma, Munich, Germany
BSA (bovine serum albumin)	Sigma, Munich, Germany
Coom. G250 Brilliant Blue	Serva, Heidelberg, Germany
Cell culture plasticware	Greiner, Solingen Nunc, Denmark Falcon, UK
DNA-size marker	Eurogentec, Cologne, Germany
DTT	Sigma, Munich, Germany
EDTA	Baker Chemicals, Deventer, NL
Ethidium Bromide	Sigma, Munich, Germany
Formamide	Fluka AG, Neu-Ulm, Germany
G418 (Geneticin)	Gibco BRL, Eggenstein, Germany
Glutathione-Sepharose	Pharmacia, Freiburg, Germany
HEPES (4-(2-hydroxyethyl)-1 -piperazine-ethanesulphonic acid	BIOMOL, Hamburg, Germany
Hybond-N (Nylon membrane)	Amersham-Buchler, Braunschweig, Germany
Lipofectamine®	Gibco, Eggenstein
L-Glutamine	Gibco BRL, Eggenstein, Germany
Kanamycin	Roche, Mannheim, Germany
Mineral oil	Sigma, Taufkirchen
MTT (3-[4,5-Dimethyltiazol-2-yl]-2,5vDiphenyltetrazoliumbromid)	Sigma, Taufkirchen
Nucleotides and Dideoxynucleotides	Pharmacia, Freiburg, Germany
PEG (polyethylene glycol)	Sigma, Munich, Germany
Protein A sepharose 4B	Pharmacia, Freiburg, Germany
Permafluor®	Molecular Probes, Leiden, Netherlands
SDS	Roth, Karlsruhe, Germany
Sodium Azide	Serva, Heidelberg
Sodium Fluoride	Sigma, Taufkirchen

Sodium Orthovanadate	Aldrich, Steinheim
Phenol	Roth, Karlsruhe
PMSF (Phenylmethy-lsulfonylfluoride)	Sigma, Taufkirchen
Polybrene (Hexadimethrine bromide)	Sigma, Taufkirchen
Poly-Lysine	Sigma, Taufkirchen
Ponceau S	Sigma, Taufkirchen
Sterile filter 0.22 µm	Nalge Company, USA
Sterile filter 0.45 µm	Nalge Company, USA
TEMED	Serva, Heidelberg, Germany
Triton X-100	Serva, Heidelberg
Tryptone®	Difco, Detroit, USA
Tween 20	Sigma, Taufkirchen
Tween 40	Sigma, Taufkirchen
Triton X-100®	Roth, Karlsruhe, Germany
Whatmann 3MM paper	Whatmann, USA
Xylol	Merck, Darmstadt
X-Omat	Kodak, Stuttgart, Germany
Yeast extract	Difco Laboratories Detroit, USA

Unless otherwise stated chemicals were purchased from Merck, Darmstadt or from Sigma, Munich.

3.2.2 Restriction, polymerases and modifying enzymes

Calf intestinal phosphatase	Roche, Mannheim, Germany
T4 DNA Polymerase	Roche, Mannheim, Germany
Klenow fragment	Roche, Mannheim, Germany
T4 DNA Ligase	Roche, Mannheim, Germany
RNAse A	Roche, Mannheim, Germany
rTaq® DNA Polymerase	Takara, Kyoto, Japan
Restriction Enzymes	New England Biolabs, Schwalbach, Germany

3.2.3 Radiochemicals

[α-^{33}P]-dATP, [γ-^{32}P]-ATP	>5000 Ci/mmol
[α-^{35}S]-dATP	>1000 Ci/mmol

All radiochemicals were purchased from NEN, Brussels, Belgium.

3.2.4 Commercial kits

Qiagen Plasmid Mini Kit, Qiagen GmbH, Hilden, Germany
Qiagen Plasmid Maxi Kit, Qiagen GmbH, Hilden, Germany
Qiaex II Gel Extraction Kit, Qiagen GmbH, Hilden, Germany
ECL Kit, NEN, Brussels, Belgium

Micro BCA Protein Assay Kit, Pierce, USA
Poly Prep® Chromatography Columns, Bio-Rad, Munich

3.3 Media and cell culture conditions

3.3.1 Medium for *E.coli* culture

The base for all bacterial culture unless otherwise stated was LB medium[48], which consisted of 1% tryptone, 0.5% yeast extract and 0.5% NaCl, pH 7.2. Ampicillin was used for selection at a concentration of 50 μg/ml. All liquid medium was solidified with 1.5% bacto-agar in order to make culture plates.

3.3.2 Bacterial culture conditions

The *E.coli* strains XL1-Blue and CJ236 were cultured at 37°C either on selection plates or as liquid medium in Erlenmeyer flasks.

3.3.3 Cell culture media

Cell culture media as well as additional components were purchased from Gibco BRL, Eggenstein, Germany, with the exception of foetal calf serum that was purchased from Sigma. In the case of the NIH3T3, HEK293, COS-7 and A431 cell lines, Dulbecco's Modified Eagle Medium (DMEM) with 4.5 mg/ml Glucose, 10% FCS, 2 mM Glutamine, 1 mM sodium pyruvate was used. All other mentioned cell lines were cultured in medium recommended by the supplier, and where possible, purchased from Gibco BRL (Germany). Selection of stably integrated cell lines was with medium containing G418 at a concentration of 1 mg/ml. Cells were frozen for storage in 90% FCS, 10% DMSO.

3.4 Stock solutions and frequently used buffers

The stock solutions listed below were all prepared with ddH_2O. "x" refers to the fold concentration of the stock solution.

BBS (2 x)	50	mM	BES
	280	mM	NaCl
	1.5	mM	Na_2HPO_4
			pH 6.96 (NaOH)
DNA-loading buffer (6 x)	0.25	%	Bromophenol Blue
	0.25	%	Xylene cyanol
	30	%	Glycerol
	100	mM	EDTA pH 8.0
Src-kinase assay buffer	20	mM	HEPES pH 7.4

1	mM	$MnCl_2$
1	mM	DTT
100	µM	Na-Orthovanadate
50	µM	ATP

Laemmli-buffer (2 x)	187.5	mM	Tris-Cl pH 6.8
	6	%	SDS
	30	%	Glycerol
	0.01	%	Bromophenol Blue
	5	%	2-ME
mRNA binding buffer	0.5	M	NaCl
	10	mM	Tris-Cl pH 7.5
	1	mM	EDTA
	0.5	%	SDS
mRNA elution buffer	10	mM	Tris-Cl pH 7.5
	1	mM	EDTA
	0.2	%	SDS
NET/gelatine (1 x)	150	mM	NaCl
	5	mM	EDTA
	50	mM	Tris
	0.05	%	Triton X-100
			pH 7.4 (HCl)
	25	mg/ml	Gelatine
PBS		13.7 mM	NaCl
		2.7 mM	KCl
		80.9 mM	Na_2HPO_4
		1.5 mM	KH_2PO_4, pH 7.4 (HCl)
PBG		0.5 %	BSA
		0.045 %	Fish gelatine
		in 1x PBS	
RIPA lysis buffer		1 %	NP-40
		1 %	Na-deoxycholate
		0.1 %	SDS
		150 mM	NaCl
		50 mM	Tris-Cl pH 7.5
		50 mM	NaF
		2 mM	EDTA
		1 mg/ml	Aprotinin
		100 mM	Na-Orthovanadate
SD-Transblot		50 mM	Tris-Cl pH 7.5
		40 mM	Glycine
		20 %	Methanol
		0.004 %	SDS

TAE (10 x)	400	mM	Tris/Acetate
	10	mM	EDTA
			pH 8.0 (Glacial Acetic Acid)
TE	10	mM	Tris/HCl pH 8.0
	1	mM	EDTA pH 8.0
Tris-Glycine-SDS (10 x)	248	mM	Tris/HCl pH 7.5
	1918	mM	Glycine
	1.0	%	SDS
Triton X-100 Lysis buffer	50	mM	HEPES pH 7.5
	150	mM	NaCl
	1.5	mM	$MgCl_2$
	200	mM	EGTA
	50	%	glycerol
	0.1	%	Triton X-100®
	1	mg/ml	Aprotinin
	100	mM	Na-Orthovanadate

3.5 Bacterial strains

The following two E.coli strains were used in this work for cloning and *in vitro* mutagenesis of plasmid DNA, respectively.

Strain	Genotype	Reference
E.coli CJ236	dut-, ung-, thi-, relA-	Kunkel, 1985[49]
E.coli XL-1 Blue	F':Tn10 proA+B+ laclq D(lacZ) M15/ RecA1 end/A1 gyr 96 (Nalr) thi hsdR17 (rk-mk+) supE44	Stratagene, USA

3.6 Cell lines

Line	Origin	Reference
A431	Human epidermoid carcinoma	ATCC CRL-1555
BOSC	Ecotropic retroviral producer line derived from HEK293	Pear (1993) [50]
COS-7	Immortalised African green Monkey kidney fibroblasts	ATCC CRL-1651

COS-7/SIRP α1	COS-7 overexpressing hSIRP α1	This work
HEK293	Human transformed kidney fibroblasts	ATCC CRL-1573
Jurkat	Human T-cell leukaemia	J. Schlessinger
K562	Chronic Myeloid Leukaemia	ATCC CCL-243
KG-1	Human myeloid leukaemia	ATCC CCL-246
MCF7	Human breast adenocarcinoma	ATCC HTB-22
MCF-10A	Human mammary gland	ATCC CRL-10317
MEG-01	Human megakaryoblastic leukaemia	ATCC CRL-2021
Melanocytes	Human	Clonetics,MD, USA
NIH3T3 cl.7	Immortalised Mouse Fibroblasts	ATCC CRL-1658
X123	Oligoclonal NIH3T3-derived cell line selected for integrated pIRES	this work
SD469	Oligoclonal NIH3T3-derived cell line overexpressing SIRP β and DAP12	this work
NT2	Human testicular teratocarcinoma	Stratagene
Raji	Human Burkitt's lymphoma	ATCC CCL-86
U-937	Human histiocytic carcinoma	ATCC CRL-1593

3.7 Plasmids and oligonucleotides

3.7.1 Cloning vectors and constructs

3.7.1.1 Cloning Vectors

Vector	Attributes	Reference
pBluescript	ColE1 ori, Amp^r, LacZ, f1 (+) IG, SK (MCS)	Stratagene, USA
pcDNA3	Eukaryotic expression vector, Amp^r, Neo^r, CMV Promoter, BGH poly A, F_1(-) ori	Invitrogen, USA
pCEP4	Eukaryotic Expression Vector, Amp^r, Hyg^r, CMV promoter, EBNA-1 gene, TK poly A	Invitrogen, USA
pLEN	Retroviral expression vector, Amp^r, Neo^r, ori from pBR322, 5'-LTR and 3'-LTR from MoMuLV, ECMV pIRES sequence.	Miller and Rosman, 1989 [51]
pLXSN	Retroviral expression vector, Amp^r, Neo^r, ori from pBR322, 5'-LTR and 3'-LTR from MoMuLV, SV40 Promoter	Adam, 1991 [52]
pIRES	Retroviral expression vector, Amp^r, Neo^r, ori from pBR322, 5'-LTR and 3'-LTR from MoMuLV, SV40 Promoter, ECMV IRES	Derived from pLXSN and pLEN (this work)
pSj26 (mod)	Eukaryotic expression vector, Amp^r, Promoter, BGH poly A, Enterokinase cleavage site between GST and fused protein, PKA labelling site	Derived from CMV pCDNA3 (this work)
pCDNA3 IgGFc	Eukaryotic expression vector, Amp^r, Promoter, BGH poly A,	Derived from CMV pCDNA3

		(this work)
pEGFP	Eukaryotic GFP fusion expression vector, Kan^r	Invitrogen, USA
pGEX-2TK	Bacterial GST fusion protein expression vector , Amp^r,	Pharmacia, Freiburg, Germany
pLXSN EKS	Derived from pLXSN, modified to contain pBluescript KS MCS.	J.Ruhe (this department)

3.7.1.2 Eukaryotic expression constructs used in this work

Name	Expressed insert
pSj26(mod)/SIRP α1 ex	hSIRP α1 ex (aa's 1-373) -GST fusion
pSj26(mod)/SIRP α2 ex	hSIRP α2 ex (aa's 1-373) -GST fusion
pSj26(mod)/SIRP β1 ex	hSIRP β1 ex (aa's 1-369) -GST fusion
pSj26(mod)/DAP12	complete DAP12 Δstop (aa's 1-113)-GST fusion
pCDNA3 IgGFc/SIRP α1 ex	hSIRP α1 ex (aa's 1-373) -IgG fusion
pCDNA3 IgGFc/SIRP α2 ex	hSIRP α2 ex (aa's 1-373) -IgG fusion
pCDNA3 IgGFc/SIRP β1 ex	hSIRP β1 ex (aa's 1-369) -IgG fusion
pCDNA3/SIRP α1	complete hSIRP α1 (aa's 1-503)
pCDNA3/SIRP α1/Y/F	hSIRP α1 Y429F Y452F Y468F Y495F (SIRP α-4Y [1])
pCDNA3/SIRP β1	complete hSIRP β1 (aa's 1-398)
pCDNA3/Syk	complete hSyk (aa's 1-635)
pCDNA3/Syk K/R	hSyk (K402R) =kinase inactive
pCDNA3/DAP12	complete DAP12 (aa's 1-113)
pCDNA3/c-src K/R	complete c-src K297M, chicken, kinase inactive
pCDNA3/c-fyn K/R	complete c-fyn K299A, chicken, kinase inactive
pLXSN/SIRP α1	complete hSIRP α1 (aa's 1-503)
pLXSN/SIRP β1	complete hSIRP β1 (aa's 1-398)
pLXSN/DAP12	complete DAP12 (aa's 1-113)
pIRES/SIRP β1+DAP12	complete SIRP β1 and DAP12 expressed bicistronically
pCEP4/Syk	complete hSyk (aa's 1-635)
pCEP4/Syk K/R	hSyk (K402R) =kinase inactive

3.7.2 Important oligonucleotides used

Sj26(mod) f 5' - CCG CTC GAG CGT CGT GCA TCT GTT GAT GAT GAT GAT AAG ATG TCC CCT ATA CTA GG -3'

Used for PCR amplification of GST from pGEX 2TK (Pharmacia, Freiburg, Germany)

Sj26 rev 5' - GCG GGG CCC TTA TTT TGG AGG ATG GTC G -3'
For PCR amplification of GST from pGEX 2TK (Pharmacia, Freiburg, Germany)

h IgG1 f 5' - CCG CTC GAG ATG GTC ACC GAC AAA ACT C -3'
For PCR amplification of human IgG1 heavy chain coding region from pBSSK-CH_2CH_3 [53]

h IgG1 r 5' - GCG GGG CCC TCA TTT ACC CGG AGA CAG -3'
See *h IgG1 f*

GP fo 5' - CCG CTC GAG ATG GTG AGC AAG GGC GAG -3'
For PCR amplification of GFP coding region from pEGFP (Invitrogen, USA)

GP rev 5' - GCG GGG CCC TTA CTT GTA CAG CTC GTC C -3'
See *GP fo*

SIRP α f 5' - GCG GAA TTC GCC ACC ATG GAG CCC GCC GGC -3'
For amplification of coding region of human SIRP α1 or 2 from cDNA [1]

SIRP α r 5' - CGG GGA TCC TCA CTT CCT CGG GAC CTG G -3'
See *SIRP α f*

SIRP α1ex rev 5' - CGG GGA TCC CCG TTC ATT AGA TCC AGT GTT CTC -3'
Used with *SIRP α f* for amplification of the extracellular domain of SIRP α1 or 2

Beta 1 f 5' - GCG GAA TTC GCC ACC ATG CCC GTG CCA GCC TCC -3'
For amplification of human SIRP β1 from plasmid [54]

Beta r stop 5' - CCG CTC GAG TCA GGC CTT CTG TTT CCA GCA GAT -3'
Reverse primer used with *Beta 1 f*

Beta ex r 5' - CCG CTC GAG AGC AGT AGG AGC CAG CGC -3'
Used with *Beta 1 f* for amplification of the extracellular domain of SIRP β1.

DAP12 f Eco R1 5' - GCG GAA TTC GCC ACC ATG GGG GGA CTT GAA CCC TGC -3'
For amplification of human DAP12 from cDNA

DAP12 Flag r Xho1 5' – GCG CTC GAG TCA CTT ATC ATC ATC ATC TTT GTA ATA CGG CCT CTG TG -3'
Used together with *DAP12 f Eco R1* (adds Flag epitope tag)

DAP12 rev nostop 5' - GCG CTC GAG CTT ATC ATC ATC ATC TTT GTA ATA CGG CCT CTG -3'
Used with *DAP12 f Eco R1* to produce DAP12 for cloning into pSj26 (mod)

DAP12 f not1 5' - GCG GCG GCC GCG CCA CCA TGG GGG GAC TTG AAC C -3'

For amplification of human DAP12 from cDNA for cloning into pIRES

DAP12 Flag r BstX1 5' - GCG CCA CCG CGG TGG TCA CTT ATC ATC ATC ATC TTT GTA ATA C -3'
Used with *DAP12 f not1*

h Syk f 5' - GCG GAA TTC GCC ACC ATG GCC AGC AGC GGC ATG GC -3'
Amplification of human Syk from cDNA

mouse Syk f 5' - CCG CTC GAG TCA GGC CTT CTG TTT CCA GCA GAT -3'
For amplification of mouse Syk from cDNA (used with *h Syk r*)

h Syk r 5' - GGC CTC GAG TTA GTT CAC CAC GTC ATA GTA G -3'
See *h Syk f* and *mouse Syk f*

h Syk r Flag 5' - GGC CTC GAG TTA CTT ATC ATC ATC ATC CTT GTA ATC GTT CAC CAC GTC ATA GTA G -3'
Used with *h Syk f* for production of Flag epitope-tagged Syk

h Syk k/R rev 5' - GTT TTT CAG GAT CCT CAC AGC CAC GGT -3'
Used for production of kinase inactive Syk by *in vitro* mutagenesis

4 Methods

4.1 Molecular Biology

4.1.1 Preparation and Purification of Plasmid DNA from Bacteria

The procedure was based, with modifications, on the alkaline lysis method described by Birnboim & Doly (1979) [55].

4.1.2 Digestion of DNA with Restriction Endonucleases

Restriction digests utilised the pre-made buffers accompanying the respective enzymes as recommended by the supplier. In a typical analytical digest, 1 µg of plasmid DNA was cut with 0.5 units of a given enzyme for a duration of 60 min at 37°C. Where necessary the enzyme was stabilised by addition of BSA (100 µg/ml).

4.1.3 Dephosphorylation of 5´ Termini

The removal of 5´ phosphate groups from DNA was necessary in order reduce the efficiency of intramolecular ligation, for example to lower the efficiency of vector self-ligation where cloning DNA fragments. Restricted DNA was therefore incubated with one unit of CIP for 30 minutes at 37°C, and the DNA purified by agarose gel electrophoresis.

4.1.4 Blunt Ending 3´ or 5´ Overhangs

It was occasionally necessary during cloning strategies to blunt end 3´ or 5´ overhangs. This was achieved using T4 DNA Polymerase. DNA possessing cohesive ends was added to 100 µM of dNTP, 1-2 U polymerase/µg DNA and incubated at 12°C for 20 minutes. Heat inactivation followed at 75°C for 10 minutes.

4.1.5 Preparation of total RNA

1g of mammalian cells from a monolayer culture were lysed and homogenised in 10 ml of a solution of 4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarkosyl 0.1 M 2-ME and 10 mM EDTA. To this mixture, 1 ml 2M Na-acetate was added, and the mixture vortexed. 10 ml water-saturated phenol was then added before the mixture was again vortexed. The sample was centrifuged at 10,000 x g for 10 minutes. The upper, aqueous phase was transferred to a fresh tube and 10 ml isopropanol was added. After a centrifugation step of 10 minutes at 3000 x g, the supernatant was discarded and the pellet redissolved in 2 ml 4 M LiCl. The sample was again centrifuged at 3000 x g for 10 minutes and the pellet redissolved in 2 ml of a solution of 10 mM Tris-Cl, pH 7.5, 1mM EDTA and 0.5%

SDS. The solution was vortexed with 2 ml chloroform and centrifuged once more for 10 minutes at 3000 x g. Finally, the upper aqueous phase was precipitated with the addition of 6 ml absolute ethanol in the presence of 0.3 M Na acetate, pH 6.0. To the pellet after a final centrifugation step of 10 minutes at 3000 x g, RNA was dissolved in an appropriate volume of TE.

4.1.6 Isolation of mRNA

A Pasteur pipette was baked and silanised, and siliconised glass wool was added to the narrow end to act as a filter. 1 ml of a 25 mg/ml suspension of oligo-dT cellulose (Stratagene, USA) was added to the pipette and washed with 20 ml of binding buffer. 1 mg of total RNA was heated for 5 minutes at 70°C in mRNA binding buffer, and applied to the column. The flowthrough was collected and added once again to the column, until the sample had run through the pipette four times to allow for sufficient binding, and the column was then washed repeatedly with binding buffer to remove unbound RNA. Elution was with 1.6 ml mRNA elution buffer. The mRNA was precipitated by the addition of 160 µl 3 M NaOAc pH 6.0 in a silanised Corex tube, and the pellet was resuspended in ddH2O to an approximate concentration of 1 mg/ml.

4.1.7 Synthesis of cDNA from mRNA

The synthesis of both cDNA strands in the production of cDNA libraries was achieved by the use of Avian Myeloblastosis Virus (AMV) Reverse Transcriptase. Two micrograms of template was added to a reaction buffer containing 50 mM Tris-Cl pH 8.3, 8 mM $MgCl_2$, 1 mM DTT, 30 mM KCl 10 mM dNTPs and 3 µG random primer $p(dN)_6$ (Roche, Mannheim, Germany) supplemented with 50 U of AMV Reverse Transcriptase. The reaction was allowed to continue for 1 hour at 37°C.

4.1.8 Polymerase chain reaction (PCR)

4.1.8.1 Amplification of RNA and DNA fragments by PCR

The basic reaction comprised 30 cycles, the first of which was preceded by an initial 3 minute denaturation step at 94°C during which time Taq polymerase was added to the reaction mixture. Each cycle consisted of a denaturation step at 94°C for 1 minute, an annealing step for 1 minute at a temperature derived individually from the expected melting temperature of the oligonucleotides used (see below) and an elongation step for 1 minute at 72°C. The program ended with a final extension step at 72°C for 5 minutes.
PCR was carried out in a final volume of 50 µl containing 10 ng linearised template, 0.2 mM dNTPs, 1 mM $MgCl_2$, 10 pmoles 'forward' primer, 10 pmoles 'reverse' primer, 1x polymerase buffer (20 mM Tris-Cl pH 8.0, 0.1 mM KCl 20 mM

DTT, 0.1 mM EDTA, 0.5% Nonidet P40 (v/v), 0.5% Tween 20 (v/v)) and 0.5 U Polymerase, Erlich (1991)[56].

4.1.8.2 Calculation of primer annealing temperature

The annealing temperature of the reaction was calculated as:

$T = T_{ml}-5$

Where T_{ml} was the T_m of the oligonucleotide with the lowest melting temperature as calculated below.

The melting temperature of every oligonucleotide primer was established according to the equation:

$$T_m = 2((A+T)+2(G+C))$$

(Where A, C, G, and T correspond to the number of each respective deoxyribonuclotides in the oligonucleotide.)

The equation neglects the effect of salt but was taken as a useful approximation of optimal T_m.

4.1.9 Site-directed mutagenesis of DNA

In Vitro Mutagenesis of DNA was performed according to Messing (1983) and Kunkel (1985) as follows:

4.1.9.1 Production of uracil-containing single stranded M13 DNA

5 ml LB medium were inoculated with 100 µl CJ236 overnight culture and incubated at 37°C with agitation until early log phase. This culture was then infected with 10^8 M13 plaque-forming units. The culture was incubated overnight at 37°C with agitation. 1.2 ml of culture was then transferred to a microfuge tube and centrifuged for one minute. The supernatant was transferred to a second microfuge tube, centrifuged and again transferred to a fresh tube. The phage particles were precipitated from the lysate by incubation at room temperature for 20 minutes after the addition of 300 µl 20% PEG/NaCl. The mixture was microfuged at full speed and the supernatant discarded. The phage sediment was resuspended in 300 µl TE10/1 buffer protein was removed by the addition of 200 µl of water-saturated phenol/chloroform. The mixture was vortexed strenuously and centrifuged at full speed in a microfuge for one minute. The upper, aqueous phase containing the single-stranded DNA was retrieved and precipitated by the addition of 200 µl 7.5 M NH_4Ac (pH 7.5) and 800 µl absolute ethanol, incubating at –70°C for 10 minutes then microcentrifuging at full power for 20 minutes. The pellet was washed in 70% ethanol to remove salt, allowed to air dry and resuspended in 20 µl

H_2O. The quality of the single-stranded DNA was analysed by visualisation on a 1% agarose gel.

4.1.9.2 Site-directed mutagenesis

Hybridisation of phosphorylated oligonucleotide was effected using the following procedure: 200 ng of single-stranded DNA and 2-3 pmol of phosphorylated oligonucleotide in hybridisation buffer (2 mM Tris-Cl pH 7.4, 0.2mM $MgCl_2$, 5 mM NaCl) in a total volume of 10 µl were incubated at 70°C for 2 minutes, then allowed to cool to room temperature. The mixture was transferred to ice and the following components were added: 1 µl 10 x Synthesis buffer (5mM dNTP mix, 100 mM Tris-Cl pH 7.5, 50 mM $MgCl_2$, 20 mM DTT), 5 E T4-DNA Ligase (1µl), 1 µg T4-Gen 32 Protein (0.5µl) and 3 E T4-DNA polymerase. The reaction mixture was incubated for 5 minutes on ice, then for 5 minutes at 25°C and finally for 90 minutes at 37°C after which time the reaction was stopped by the addition of 80 µl of TE 10/1. 10 µl was used to transform competent *E.coli* bacteria.

4.1.10 DNA gel electrophoresis

The separation of DNA fragments, that were all between 250 bp and 14 kB was achieved using agarose gel electrophoresis, Sambrook (1989) [48]. Unless otherwise stated 1.2% agarose was used for all preparative and analytical gels. The DNA to be separated was administered in 1x DNA-loading buffer. The gel was visualized after staining with ethidium bromide using a UV_{365nm} light source.

4.1.11 Extraction of nucleic acids from agarose

Following restriction, DNA was electrophoresed in an agarose gel. Agarose containing a restriction fragment of interest was excised and weighed. Three volumes of solubilisation buffer (QIAEX II Gel extraction kit, QIAGEN) were added together with 10 µl of silica particles. The sample was vortexed and incubated at 50°C for 10 min. The sample was then centrifuged and the pellet washed with 500 µl of solubilisation buffer. After subsequent centrifugation the pellet was washed twice with 500 µl of a high salt-ethanol wash buffer and air-dried for 2 min. Elution of DNA from the silica beads was carried out by adding 20 µl of 10 mM Tris-Cl, pH 8.5 and incubating for 5 minutes at 50°C. Centrifugation ensured that the eluate was free of silica for subsequent manipulations.

4.1.12 Purification of PCR products

PCR products were electrophoresed in an agarose gel and the product was excised and purified using the QIAEX II Gel Extraction Kit according to the manufacturers recommendations.

4.1.13 Desalting and concentration of nucleic acids

Where more than one step was required to successfully modify DNA, it was often necessary to change the buffer conditions, especially where downstream enzymatic reactions were sensitive to residual salt. This was achieved by precipitation in absolute ethanol followed by a wash step with 70% ethanol and air-drying. The same method was also used to concentrate DNA samples.

4.1.14 Cloning of DNA

4.1.14.1 Ligation of DNA fragments

DNA ligation was performed according to the method published by King & Blakesley (1986) [57].

4.1.14.2 Preparation of competent *E.coli*

Preparation of competent cells was according to Inoue et al. (1990)[58]

4.1.14.3 Transformation of plasmid DNA into competent E.coli

50 μl of suspension of competent E.coli prepared using the above protocol was added to 10 ng of supercoiled plasmid (prepared by the alkaline lysis procedure) or 5 μl of ligation reaction. The mixture was incubated on ice for 20 minutes, rapidly transferred to a water bath preheated to 42°C for 50 seconds and immediately placed back on ice for 2 minutes. 1 ml LB medium was added, and the mixture was incubated at 37°C for one hour. After this time, 200 μl of culture was spread onto agar plates made with LB containing ampicillin (100 μg/ml). The remainder of the culture was centrifuged at low speed and the pellet resuspended in 50 μl LB. This was then spread on a second LB amp plate. The plates were incubated for 14 hours at 37°C after which time individual colonies were visible that could be collected and transferred to 4 ml LB for incubation overnight at 37°C.

4.1.15 DNA sequencing

DNA sequencing followed the method originally described by Sanger et al (1977) [59].

4.2 Protein chemistry and biochemistry

4.2.1 Preparation of mammalian cell lysates for analysis

To analyse specific cellular proteins using the techniques of protein chemistry, cell lysates were prepared with buffer containing Triton X-100. Treatment with this mild non-ionic detergent has the effect of solublising the plasma membrane, whilst

leaving cytoskeletal proteins insoluble and the cell nucleus intact. Enabling the separation of other cellular proteins from these components. Before lysis, the cells were washed twice with ice-cold PBS and an appropriate volume of Triton X-100 lysis buffer was added relative to the area of the dish (a 10 cm cell culture petridish was treated with 1 ml buffer). After 5 minutes incubation on ice, the lysates was scraped to the bottom of the dish and transferred to a microfuge tube. The tube was centrifuged at full speed and the supernatant was transferred to a new tube. Cell lysates were used immediately or stored at –70°C.

4.2.2 Electrophoretic separation of proteins by Laemmli SDS-PAGE

One-dimensional denaturing gel electrophoresis was performed as described by Laemmli (1970) [60]. Proteins were solublised by boiling in the presence of Laemmli-buffer (2 x). An aliquot of the sample was applied to a 12% acrylamide gel and electrophoresis was performed at room temperature at a constant current of approximately 1mA/cm. The gel front was allowed to migrate out of the gel before electrophoresis was discontinued. Commercially available protein standards (Sigma, Munich, Germany) enabled the estimation of apparent molecular weight.

4.2.3 Staining proteins in gels

Proteins were stained after electrophoresis by Coomassie Blue staining according to Sambrook (1989)[48].

4.2.4 Protein blotting

Protein transfer from an SDS gel to or Hybond-ECL nitro-cellulose membrane was carried out by electroblotting in a semidry electroblotter according to Ausubel (1995) [61]. Immediately after gel electrophoresis a blot-sandwich was assembled and the proteins transferred at room temperature at a constant current of 0.8 mA/cm^2 for 3-5 hours. The transfer was subsequently interrupted, the blot-sandwich disassembled and the blot stained in Ponceau S solution, allowing protein molecular weight indicators and bands of interest to be marked and transfer efficiency to be estimated. Complete destaining with NET/gelatine was necessary before the blot could be incubated with specific antibodies.

4.2.5 Immunodetection (Western blotting)

The procedure was carried out according to the procedure described by Harlow & Lane (1988)[62]. Briefly, the membrane was incubated with antibodies that specifically recognised the target protein. Polyclonal antibodies were used at a dilution of 1:1000 in NET/gelatine. Before incubating the blot with antibody, the membrane was immersed in NET/gelatine for one hour. Subsequently, the blot was incubated in NET/gelatine containing the primary antibody and gently shaken overnight at 4°C. The blot was washed again in NET/gelatine and incubated with

enzyme-antibody conjugate at room temperature for one hour. The membrane was washed three times for 20 minutes at room temperature to remove excess secondary antibody conjugate, and specific proteins were visualised by use of the ECL kit (NEN), following the manufacturers instructions.

4.2.6 Immunoprecipitation

The lysate was spun at high speed, transferred to a fresh tube and incubated on ice with 10 µl hybridoma tissue culture supernatant or 5 µl rabbit serum for 1 hour. 50 µl of protein G-bound sepharose beads was added and the lysate placed on a rotating arm for 2 hours at 4°C. The beads were pelleted by centrifugation and washed twice for 10 minutes in l ml of lysis buffer. Antigen was eluted by boiling the beads in 20 µl of 2x Laemmli-buffer.

4.2.7 Immunolocalisation

Immunofluorescence allows for an examination of the subcellular localisation of proteins. Cells are first fixed, then permeabilised with a mild detergent to allow the antibody access to subcellular compartments within the cell. Unspecific binding sites are blocked with PBG, and then the cells are immersed in a solution that contains antibody recognising the desired protein (the primary antibody). The sample is washed a second time and incubated with a second antibody that recognises the primary antibody (the secondary antibody). This antibody is coupled to a fluorophore. The emitted light of the fluorophore betrays the subcellular localisation of the target protein when examined by fluorescence microscopy while exposing the sample to light at the excitation wavelength of the fluorophore.
Adherent cell cultures were seeded on sterile, uncoated glass cover slips in a small cell culture well. At 50% confluency, the cells were washed with PBS and incubated for 30 minutes at 4°Cwith 2% Paraformaldehyde in 0.1 M Sodium cacodylate buffer, pH 7.4 (Electron Microscopy Sciences, USA). After washing 3 times in 100 mM Glycine in PBS for 10 minutes each time, the cells were immersed in 0.2% Tween 20 in 1xPBS to permeablise them and washed several times in PBG, then incubated with the primary antibody for at least two hours at RT. The best dilutions for the primary and secondary antibodies were determined empirically. The cells were washed again several times in PBG to remove residual unbound antibody and subsequently incubated with Cy3-konjugated secondary antibody (Molecular Probes, Leiden, Netherlands) for 2 hrs at RT. All stages following application of the secondary antibody were performed in the dark. Cells were finally incubated with DAPI in PBS for 10 minutes at RT, the cells washed once more and mounted upside down onto a glass slide in Permafluor® (Molecular Probes, Leiden, Netherlands). Samples were visualised and photographed with a Zeiss Axiovert X-100 Digital Confocal Microscope.

4.3 Methods in cell biology

4.3.1 General cell culture techniques

All mammalian cells were cultivated in a humidified incubator (Heraeus, B5060 Ek/CO_2) at between 5 and 7% CO_2, 37°C. All cell culture work was carried out aseptically under a laminar flow hood (Heraeus, Laminair). Cell were regularly fed and passaged with fresh medium. Cells were frozen on dry ice in freezing medium (90% FCS, 10% DMSO). Cells were counted with a Neubauer Counting Chamber (Sigma, Munich, Germany).

4.3.2 Transfection of mammalian cells with plasmid DNA

Unless otherwise indicated, plasmid DNA was introduced to mammalian cells using the calcium phosphate method [63]. Transfections carried out using Lipofectamine®(Gibco BRL, Eggenstein, Germany) adhered to the manufacturer's stipulations. Where necessary, efficiency of transfection was assessed using a parallel transfection with pCDNA3 GFP, the number of green cells showing up in fluorescence microscopy giving a satisfactory estimate of the number of positive transfectants.

4.3.3 Retroviral gene transfer into NIH3T3 cells

To produce stable NIH3T3-derived cell lines that overexpressed a particular protein, BOSC 23 cells[50] were seeded on a 10 cm cell culture dish and transiently transfected with retroviral expression vector (pLXSN or pIRES) containing as an insert the construct to be expressed. 24 hours after the transfection, the medium was replaced with 5 ml of fresh medium and left overnight to collect secreted virus particles after which time the medium was filtered through a 0.45 µm sterile filter.
For infection, exponentially growing target cells were seeded onto 6 cm cell culture dishes and allowed to spread. 3 ml of collected and filtered BOSC medium was administered to the cells in the presence of Polybrene at a concentration of 8 µg/ml. The cells were incubated at 37°C for between 4 and 16 hours. After infection the medium was changed and cultured for two weeks in medium containing G418 to obtain stable cell lines. Individual G418-resistant colonies resulting from the amplification of a single cell were isolated and cultured separately to obtain monoclonal cell lines. The expression of the target protein was usually assessed by Western blot with specific antibodies.

4.3.4 *In vitro* src-family kinase assay

This assay is based on the protocol found in Ausubel (1995)[61], with some modifications. Enolase was used as the exogenous substrate for the src-family kinase assay. It was however necessary to denature the enolase by acid treatment for it to function as an appropriate substrate. Preparation of the enolase was as follows:

100 µg of enolase suspension from rabbit muscle (Roche, Mannheim, Germany) was microfuged at full speed at 4°C. The supernatant was retained and 10 µl of a solution of 1 mM DTT, 50 mM HEPES pH 7.0 was added, and incubated for 60 minutes on ice. At this point, the enolase could be stored at –70°C for later use. An equal volume of 100 mM acetic acid was added to the enolase and the mixture was incubated for 5 minutes at 30°C, before replacing on ice. This acid-denatured enolase was used no later than one hour after preparation.
Immunoprecipitations were performed as described above, and after the last wash was aspirated, 20 µl of src kinase assay buffer containing 5 µCi of [γ-^{32}P] ATP and 1 µg of acid-denatured enolase was added to the beads. The sample was incubated for 10 minutes at 30°C after which 20 µl Laemmli sample buffer was added. The samples were separated by SDS PAGE and blotted to a nitrocellulose membrane. Western blots were carried out, if necessary at this stage, after which time the membrane was exposed to autoradiography to identify [γ-^{32}P] - labelled proteins.

4.3.5 Cell adhesion assay

The cell adhesion assay was carried out according to the method described by Lagenaur et al. (1987)[64]. Briefly, 1 µg of each respective protein was immobilised onto nitrocellulose-coated plastic dishes (35 mm) by air-drying at RT. Unspecific binding of cells to nitrocellulose was prevented by blocking with 1% BSA solution in PBS. A total of 3 x 10^5 haematopoietic cells in serum-free medium were allowed to adhere to the immobilised protein for 1 hour at 37°C. Non-adherent cells were removed by gently rinsing the dishes with warm PBS. Specific binding was evaluated under a phase contrast microscope.

4.3.6 Photometric quantification of viable (living) cells by MTT assay

To quantify viable adherent cells the method described by Mosmann (1993) [65]was used. The test measures the activity of certain mitochondrial enzymes and therefore reflects the total number of living cells. The substrate, MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is converted by these enzymes into an insoluble violet compound, which is readily measurable photometrically.
MTT was added into the medium of adherent cells in a 96-well dish to a final concentration of 0.6 mg/ml, and the cells were incubated for 2 hours at 37°C. The subsequent addition of isopropanol to 60% v/v and HCl to 12 mM lysed the cells and solublised the violet precipitate. The sample was measured at 570 nm against a reference of 690 nm.

4.4 Preparation of special expression systems

4.4.1 Preparation of new cloning vectors

4.4.1.1 pIRES vector

The pIRES cloning vector was designed for the eukaryotic expression of two constructs simultaneously by achieving bicistronic transcription via the usage of the encephalomyocarditis virus (ECMV) internal ribosomal entry site (IRES) in a retroviral vector with separate concomitant neo^r under the independent control of the SV40 promoter.
The ECMV IRES was cut from pLEN by digestion with EcoR1 and Bcl1 and subcloned into pBluescript (KS), which had been opened up by digestion with Eco R1 and Bam H1. Bcl 1 has a sticky end that is compatible with that of Bam H1 so the ligation of the insert into pBluescript destroyed both the Bcl 1 and Bam H1 site. This vector was opened by digestion with Eco R1, the ends were blunted (technique is described elsewhere here) and the vector religated to destroy the site. The insert was then again cut out from the vector by digesting with Xho 1 and Not 1 and subcloned into pLXSN EKS (produced by J.Ruhe, this department, unpublished) to give pIRES.

4.4.1.2 Production of pIRES/SIRP β1+DAP12

SIRP β1 was amplified by PCR using the two oligonucleotides *Beta 1 f* and *Beta r stop* and subcloned into pIRES at these sites. Subsequently, DAP12 was amplified from cDNA with the two oligonucleotides *DAP12 f not1*and *DAP12 Flag r BstX1* and cloned into the vector to give pIRES/SIRP β1+DAP12.

4.4.1.3 pSj26 (mod) vector

The pSj26 (mod) cloning vector was designed for the eukaryotic expression and secretion of C-terminal GST fusion proteins and was derived from the pCDNA3 cloning vector (Invitrogen) by the following procedure:
The complete DNA sequence coding for *Schistosoma japonicum* Glutathione-S-Transferase (GST) was amplified from the vector pGEX 2TK (Pharmacia Biotech, Freiburg, Germany) by polymerase chain reaction (PCR) using the oligonucleotides Sj26 *(mod) f* and *Sj26 rev* as primers. The PCR product was digested with *Xho 1* and *Apa 1* and cloned into pCDNA3 at these sites.

4.4.1.4 Production of pCDNA3 IgGFc vector

The pCDNA3 IgGFc cloning vector was designed for the eukaryotic expression and secretion of C-terminal Ig fusion proteins and was derived from the pCDNA3 cloning vector (Invitrogen) by the following procedure:

The complete DNA sequence coding for the CH_2 and CH_3 regions of the human IgG1 heavy chain was amplified from the vector pBSSK-CH_2CH_3 [53] by polymerase chain reaction (PCR) using the oligonucleotides *h IgG1 f* and *h IgG1 r* as primers. The PCR product was digested with *Xho 1* and *Apa 1* and cloned into pCDNA3 at these sites.

4.4.1.5 pSj26 (mod) or pCDNA3 IgGFc derived expression constructs

The coding region corresponding to the extracellular domain of SIRP α1,SIRP α2 or SIRP β1 was amplified from clones previously obtained[1] by PCR using the following primers:

Extracellular domain	Oligonucleotides used
SIRP α1 ex and SIRP α2 ex:	*SIRP α f* and *SIRP α1ex rev*
SIRP β1 ex:	*Beta 1 f* and *Beta ex r*

The product obtained was digested with Eco R1 and Xho 1 and cloned into either pSj26 (mod) or pCDNA3 IgGFc at these sites.
This method produced the following plasmids:

pSj26 (mod)/SIRP α1 ex
pSj26 (mod)/SIRP α2 ex
pSj26 (mod)/SIRP β1 ex
pCDNA3 IgGFc/SIRP α1 ex
pCDNA3 IgGFc/SIRP α2 ex
pCDNA3 IgGFc/SIRP β1 ex

4.4.2 Production and purification of secreted fusion proteins

The pSj26 (mod)-SIRPex or pCDNA3 IgGFc-SIRPex expression constructs were transfected into 293 cells by the Calcium Phosphate method [63]. Cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% FCS. After selection with 1 mg/ml G418 (Sigma) for 2 weeks, 10 surviving clones were tested for expression and secretion of the protein by Western blot with antibody against the extracellular domain of SIRP α that recognises both SIRP α and SIRP β [1]. A single high-expressing clone was chosen and used to produce the fusion protein. Medium was collected from confluent cultures daily. One litre of collected medium was sterile filtered and incubated with mild agitation overnight at 4˚C with either 1 ml Glutathione sepharose (Pharmacia, Freiburg, Germany) for GST fusion proteins, or 1 ml Protein A sepharose for h IgG fusion proteins. The sepharose was separated and washed with Phosphate-Buffered Saline (PBS).
For GST fusion proteins, elution was with 5 ml 10mM Glutathione for 10 minutes at 20°C.
For hIgG fusion proteins, elution was with 10 ml 100 mM Sodium citrate pH 2.2 into a solution of 1 ml 1 M Tris-Cl pH 8 to immediately neutralise the pH conditions.
Both eluents were dialysed overnight 1:10^5 in PBS/10% glycerol.

Protein concentration was determined using MicroBCA protein determination kit (Pierce, Rockford, IL).

5 Results

5.1 Extracellular domain of SIRP α1

5.1.1 Secretion of SIRP extracellular domains as GST fusions

The extracellular domain of SIRP α molecules comprises more than half of the entire molecule, is highly glycosylated and contains three (or one) immunoglobulin-like domains. It is therefore reasonable to assume that the extracellular domain of SIRP α molecules is likely to be involved in interactions with other molecules that may be ligands. In order to study this, a system was developed to produce and isolate mature, eukaryotically processed extracellular domains as fusion proteins with GST or hIgG1 fused to the C-terminal of the extracellular domain. Using this system, fusion proteins were derived from the extracellular domains of human SIRP α1, SIRP α2, which are two members of the SIRP α family subtype, as well as from the extracellular domain of SIRP β1, which is one member of the SIRP β subtype. These fusion proteins are denoted SIRP α1exGST, SIRP α2exGST, SIRP β1exGST and SIRP α1exIgG, SIRP α2exIgG, SIRP β1exIgG, respectively. The proteins were purified in sufficient abundance for use as antigens in antibody production and in adhesion assays. These proteins are depicted in Figure 4.

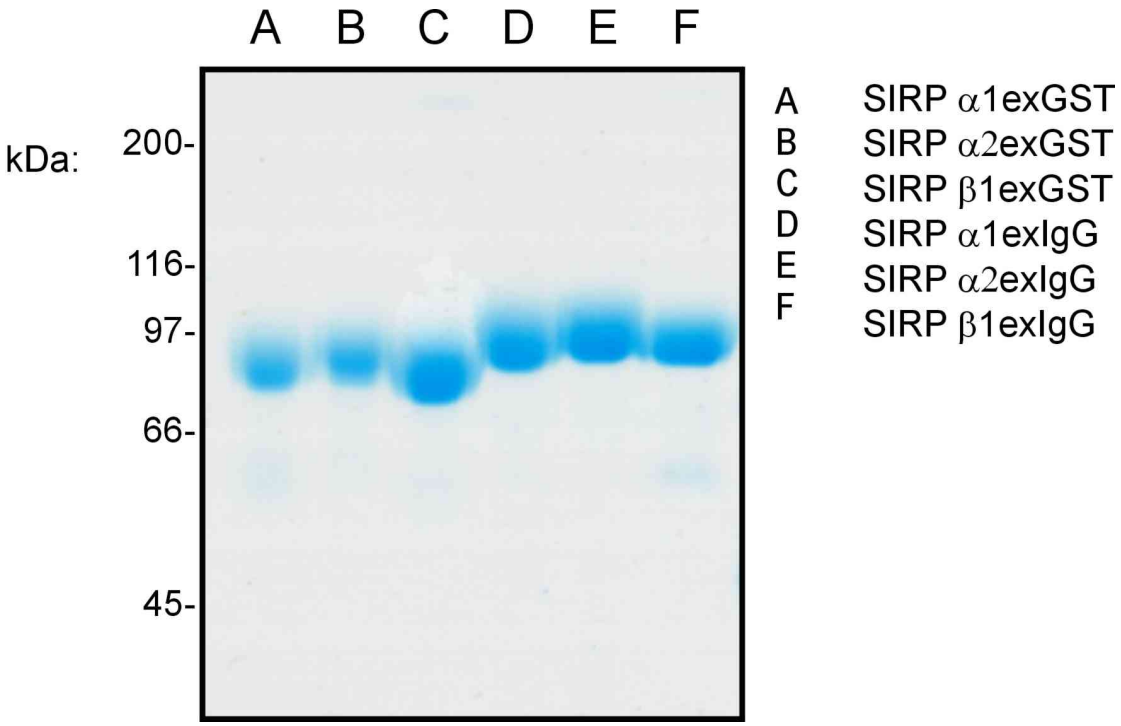


Figure 4 Affinity purified GST- and IgG- extracellular domain fusion proteins from the SIRP family. 1 µg of each purified fusion protein was separated on 10% SDS-PAGE and the gel stained with Coomassie Blue.

Figure 4 shows that the proteins were all highly pure, and that despite their high degree of similarity, SIRP α1 ex, SIRP α2 ex and SIRP β1 ex all had unique mobilities on SDS PAGE. The additional cleavage site introduced between the extracellular domain moiety and the GST tag was tested using enzymatic digestion with commercially purified enterokinase as shown in Figure 5. Although it was not necessary for the ensuing experiments, the experiment is presented as a reference for others wishing to use this secretion system.

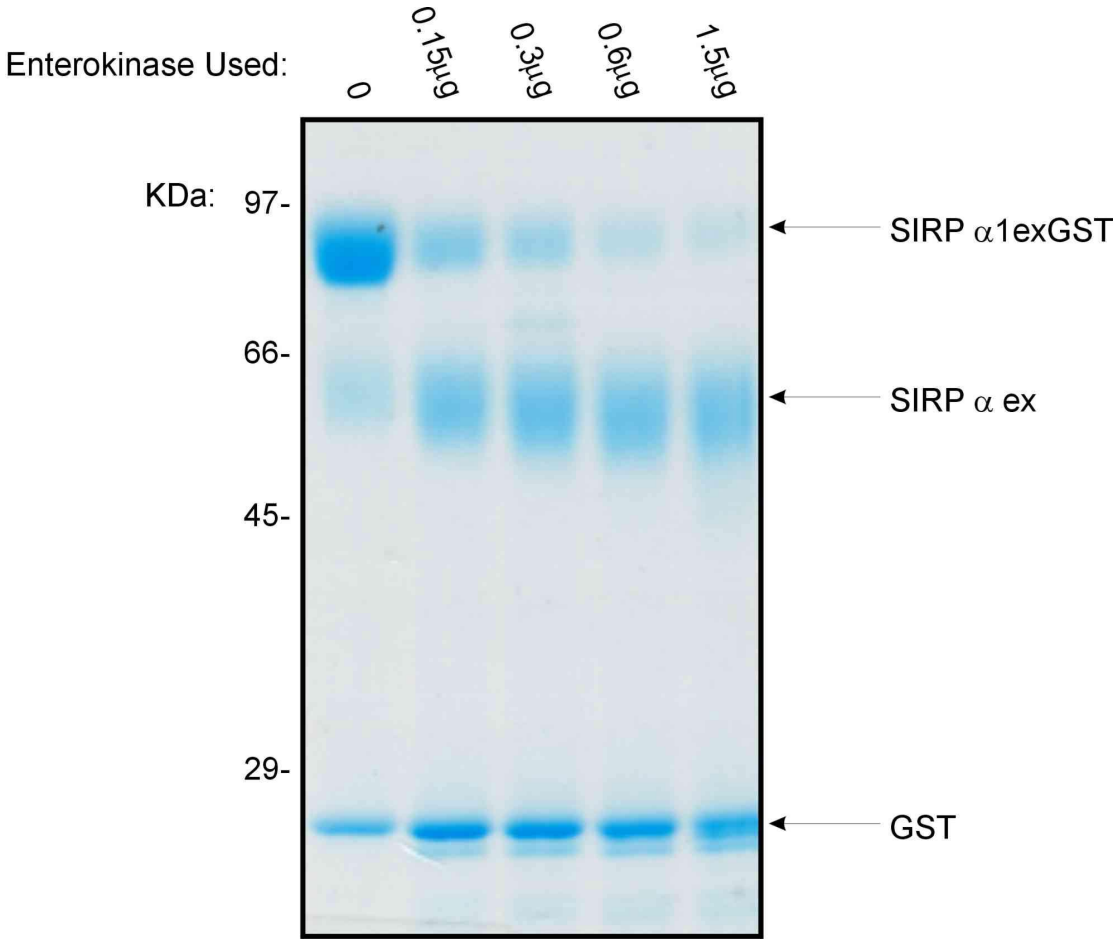


Figure 5 Cleavage of SIRP α1exGST with enterokinase. 1 µg SIRP α1exGST was digested for 20 hours at 37°C with increasing quantities of enterokinase (Roche, Mannheim). Samples were separated on 10% SDS-PAGE and bands were visualised by Coomassie Blue Staining.

Figure 5 presents an experiment to first test the sensitivity of the internal enterokinase cleavage site to cleavage with enterokinase. SIRP α1 extracellular domain liberated from the GST tag (SIRP α ex) and the GST moiety itself (GST) increase in a dose-dependent fashion while SIRP α1exGST is consequently depleted. As little as 0.15 µg are enough to produce significant cleavage of the protein, however, some latent cleavage is visible even without addition of enterokinase, possibly by cleavage by other proteases present in the extract. Complete cleavage of SIRP α1exGST is not achieved, even when as much as 1.5µg of enzyme is used. Unspecific protein degradation can also be seen at this

dosage, which is not therefore appropriate. Optimal cleavage therefore probably occurs when 0.15-0.3 µg of enzyme is used.

5.1.2 SIRP ex GST fusions as antigens for immunisation

The GST fusion protein secretion system described above is able to produce pure protein in milligram amounts, sufficient quantity for use as antigen in antibody production. Polyclonal antibodies against SIRP α1exGST and SIRP β1exGST were produced and affinity-purified using the respective antigen as a ligand. These antibodies are both sensitive and selective for their relevant antigens when compared in Western blots of crude lysates prepared from HEK293 cells that overexpress either SIRP α1 or SIRP β1 (Figure 6), although the murine homologues of SIRP α and SIRP β are not detectable (results not shown). These antisera are therefore used throughout this study except where otherwise indicated.

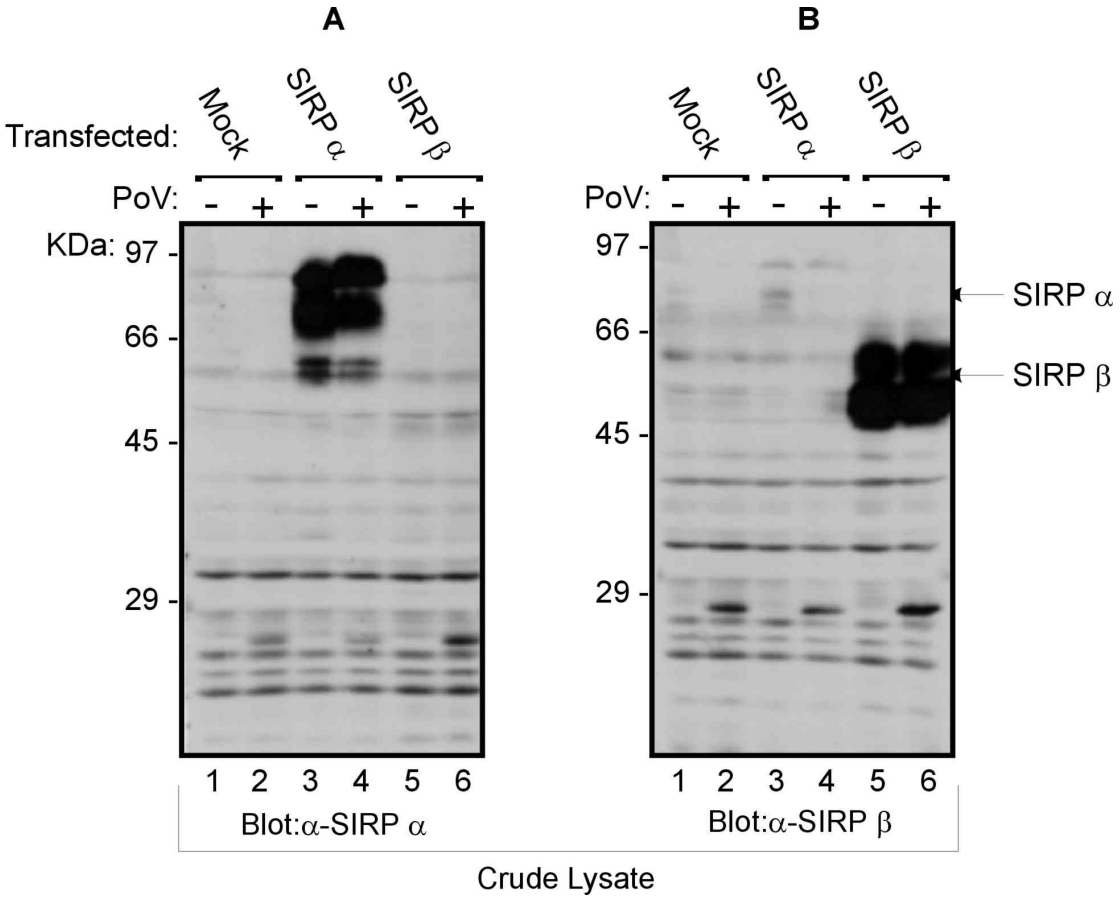
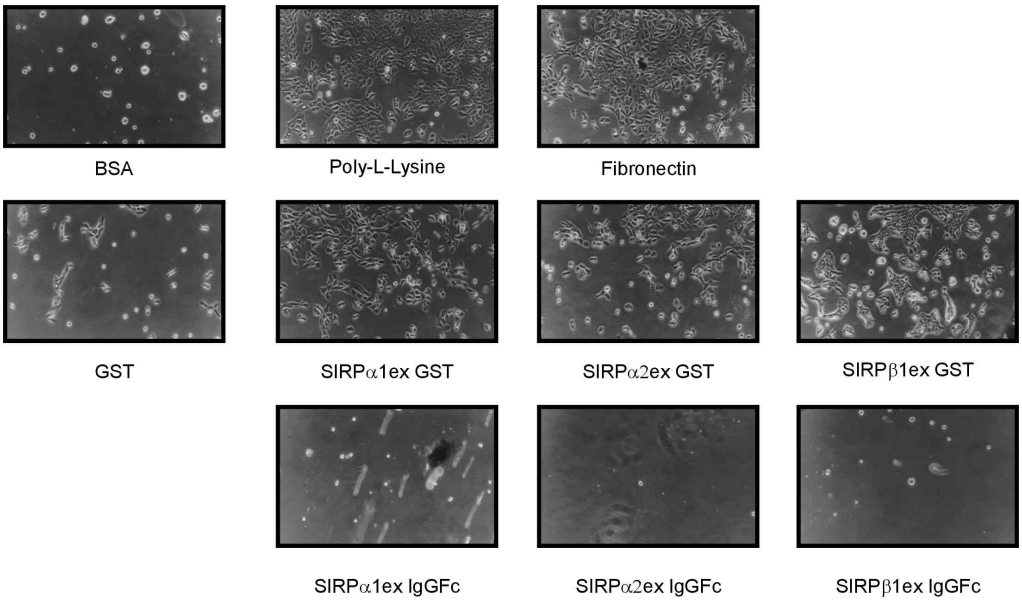


Figure 6 Comparison of the selectivity of anti-SIRP α and anti-SIRP β antisera in Western blot. Lysates prepared from HEK293 cells transiently overexpressing SIRP α1 (both pictures, lanes 3 and 4) or SIRP β1 (both pictures, lanes 5 and 6) were separated on SDS-PAGE and subjected to Western blot with affinity purified α-SIRP α (A) or α-SIRP β (B) antiserum.

5.1.3 Cell adhesion to SIRP ex GST fusions

In order to investigate the potential affinity of the extracellular domain of different SIRPs for cell surface molecules, the purified proteins were immobilised on cell culture dishes and cells of different type and lineage were dispersed over the plate and allowed to settle. The plates were washed and photographed and the degree of adhesion was approximated by eye. Eleven different cell types were examined in this way, and two typical adhesion profiles are presented in Figure 7. Results of all cell lines are tabulated in Figure 8.



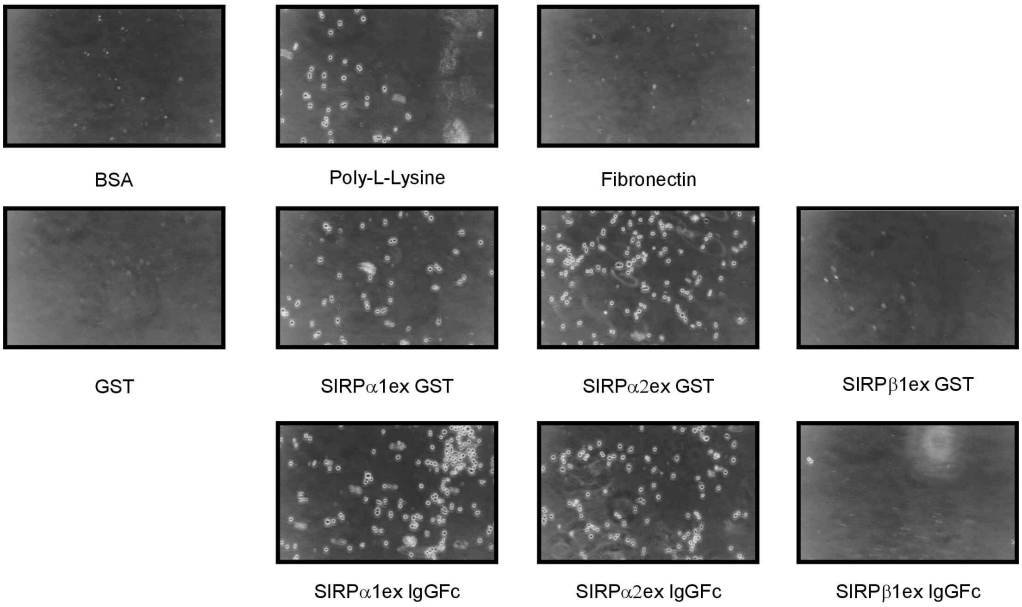


Figure 7 Adhesion profiles of two human cell lines (MCF10A and MEG-01) to an array of GST or IgG fusion proteins. Cells were dispersed over proteins spotted onto cell culture plates and allowed to settle. After gentle washing, the plates were photographed. The protein immobilised on the plate is labelled under each photograph.

Cell line	Description	BSA	Poly-L-Lysine	Fibronectin	GST	SIRP α1ExGST	SIRP α2ExGST	SIRP β1ExGST	SIRP α1ExIgG	SIRP α2ExIgG	SIRP β1ExIgG
MCF7	Breast Carcinoma	-	+++	++	-	+++	+++	-	++	++	-
U937	Histiocytic Carcinoma	-	-	-	-	+++	+++	-	+++	+++	-
K562	Myelocytic Carcinoma	-	-	-	-	+++	+++	-	+++	+++	-
Jurkat	Acute T-cell Leukaemia	-	-	-	-	++	++	-	+++	+++	-
MCF10A	Human Mammary Gland	-	+++	+++	-	+++	+++	+++	-	-	-
KG1	Acute Myelogenous Leukaemia	-	-	-	-	+++	+++	-	-	-	-
Raji	Burkitt's Lymphoma	-	+	-	-	+++	+++	-	+++	+++	-
NT2	Testicular Teratocarcinoma	-/+	+++	+++	-/+	+++	+++	-	+++	+++	-
MEG01	Megakaryoblastic Leukaemia	-	++	-	-	++	++	-	+++	+++	-
Human melanocytes		-	++	++	-	+++	+++	-	-	-	-
COS7	Transformed Monkey Kidney Cells	-	-	++	-	+++	+++	-	-	-	-

Figure 8 Comparison of eleven cell lines in adhesion assay. Adhesion assay was performed as described. Photographs were judged by eye and assigned marks representative of their relative adhesion, ranging from "–" (no adhesion) to "+++" (strong adhesion).

The following observations can be made from the table in Figure 8: Firstly, there is virtually no adhesion to GST alone, meaning that when cells adhere, it is to the SIRP extracellular domains. Secondly, all cell lines tested are able to adhere to both SIRP α1exGST and SIRP α2exGST, to different degrees. While cell adhesion varies with the cell line used, results with SIRP α1exGST are the same as with SIRP α2exGST. However, only one cell line, MCF10A, adheres to SIRP β1exGST.

There is a significant difference in cell adhesion pattern between the GST fusion proteins and their IgG fusion proteins. In seven out of the eleven cell lines tested, adhesion to SIRP α1exIgG and SIRP α2exIgG was the same as SIRP α1exGST and SIRP α2exGST. In four cases, however, there was no adhesion to the SIRP α1exIgG and SIRP α2exIgG while there was substantial adhesion to SIRP α1exGST and SIRP α2exGST.

5.1.4 CD47 (IAP) is an extracellular ligand for SIRP α

The previous result was taken to mean that many different cell types express a ligand for SIRP α molecules. A project was undertaken in collaboration with Drs. H.-J. Bühring and M. Seiffert to find a ligand for SIRP α1. In order to identify

potential ligands for SIRP α molecules, SIRP α1exGST was biotinylated and incubated with cells. Using flow cytometry, a cell line was selected for particularly high affinity for the fusion protein and therefore with a high expression level of "SIRP α-ligand". A single cell type was selected and a panel of mAb was screened to isolate an antibody that could compete with the association. One such antibody was found, "CC2C6", that recognised CD47[21].

To confirm the association of CD47 with SIRP α1, two cell lines were used for comparison: the ovarian carcinoma cell line OV10 expresses no detectable CD47 using experimental assays, including Western blot, Northern blot and RT-PCR[66]. Stable control (OV10/X) and CD47-expressing (OV10/CD47) cell lines, derived from the OV10 ovarian carcinoma cell line, were obtained and screened in the adhesion array in the manner described previously. The results of this experiment are presented in Figure 9.

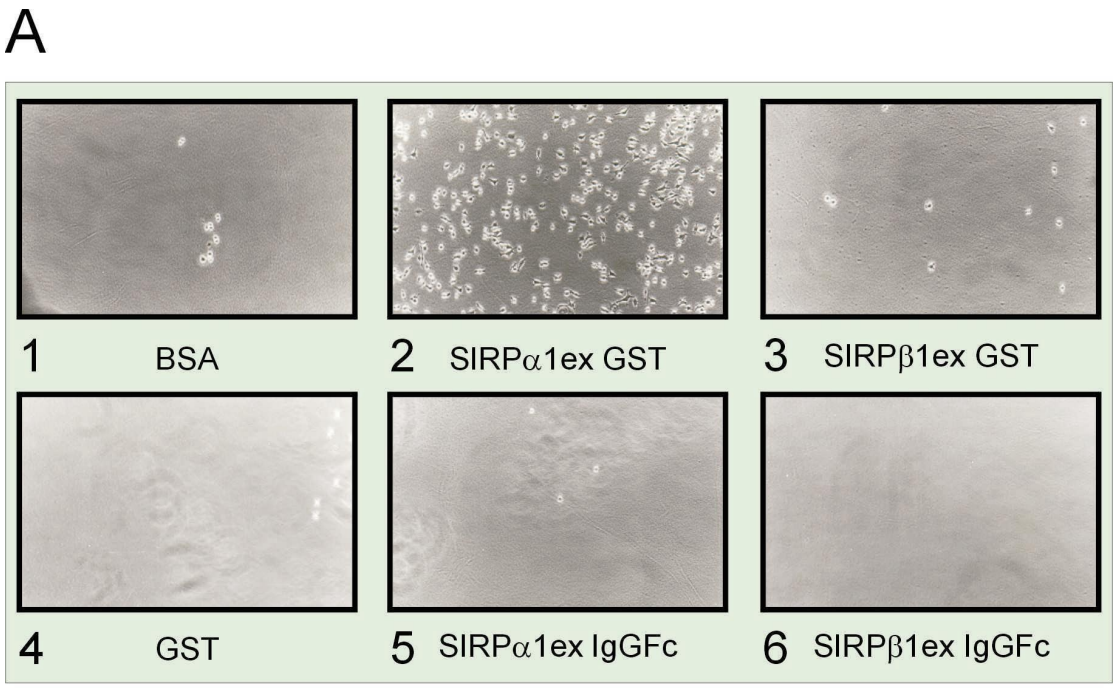


Figure 9 CD47 is a ligand for SIRP α. The picture shows a comparison of OV10 cells (OV10/X) not expressing CD47 with OV10 cells overexpressing CD47(OV10/CD47) in adhesion assay. The immobilised probe protein is labelled beneath its respective photograph.

Figure 9 shows that cells overexpressing CD47 (panel B) adhere strongly to both SIRP α1exGST and SIRP α1exIgG (plates 2 and 5). However, cells that do not express CD47 (panel A) are unable to adhere to SIRP α1exIgG (plate 2), but still adhere to SIRP α1exGST (plate 5). Neither control cells nor cells overexpressing CD47 adhere to SIRP β1exGST or SIRP β1exIgG (both panels, plates 3 and 6).

5.2 Regulation of SIRP α1 phosphorylation by tyrosine kinases

SIRP α is tyrosine phosphorylated following numerous different stimuli. It has been postulated that RTKs are directly responsible for SIRP α tyrosine phosphorylation[1]. Others have suggested that src family kinases are responsible for this tyrosine phosphorylation[39]. A431 cells, which express SIRP α and EGFR in abundance, were used as a model system to identify whether the kinase that directly phosphorylates SIRP α following EGF stimulation is either the EGFR or a cytosolic (src-family) tyrosine kinase. A431s cells were stimulated with EGF either with or without preincubation with PP1, a selective src-family kinase inhibitor.

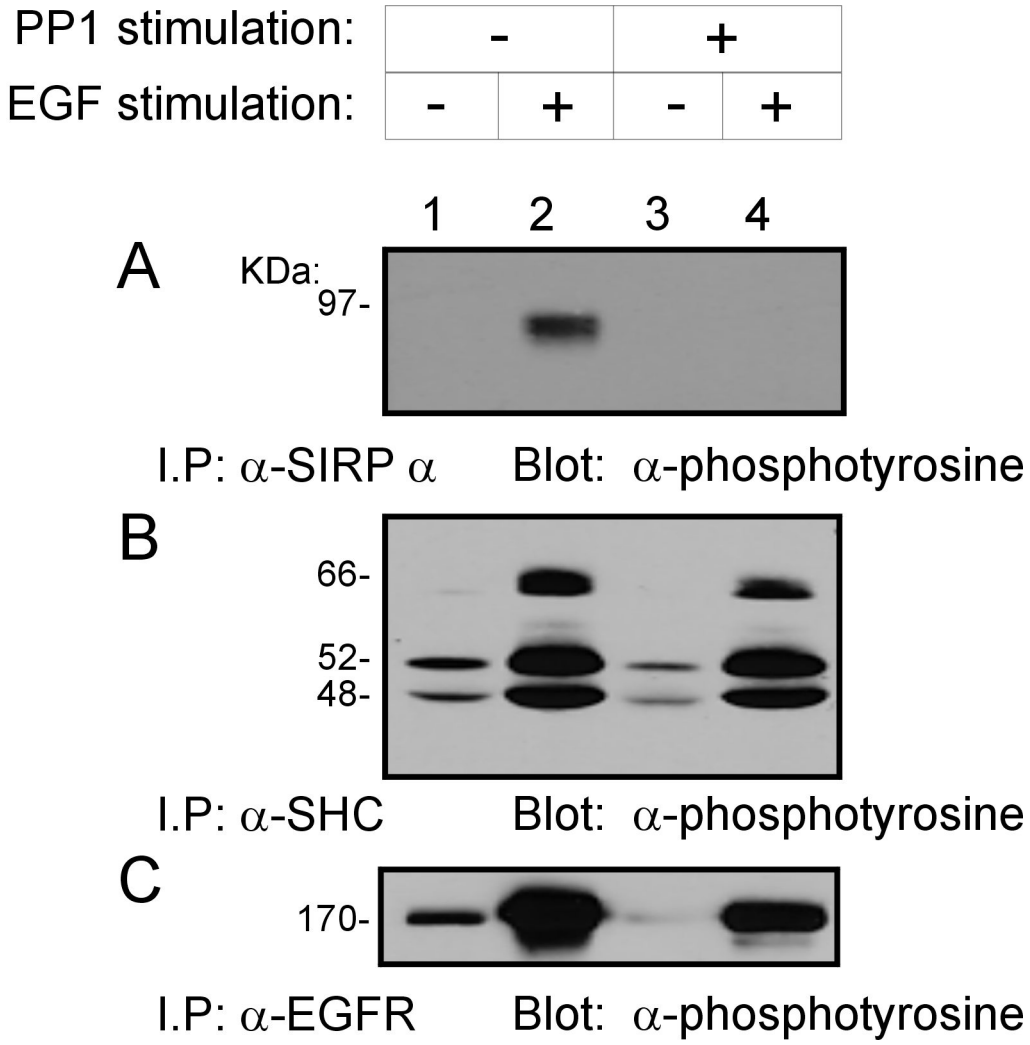


Figure 10 Treatment with the src-family kinase inhibitor PP1 inhibits EGF-induced tyrosine phosphorylation of SIRP α. A431 epithelial carcinoma cells were stimulated with 20 ng/ml EGF for 5 minutes (lanes 2 and 4) and 10 μM PP1 for 15 minutes. Cell lysates were immunoprecipitated with ExC_2 anti-SIRP α polyclonal antiserum, homemade anti-SHC antiserum or anti-EGFR mAb 108. Immunocomplexes were separated on 10% SDS-PAGE and tyrosine phosphorylated proteins were detected in Western blot with anti-phosphotyrosine antibodies.

The results shown in Figure 10 show that EGF stimulation leads to the tyrosine phosphorylation of SIRP α (panel A, compare lanes 1 and 2). However, treatment with PP1 abolishes this induced tyrosine phosphorylation. The selectivity of the inhibitor for the kinase that tyrosine phosphorylates SIRP α is illustrated in panel B, where the SHC adaptor protein, an established substrate of the EGFR, was immunoprecipitated and detected in Western blot with an anti-phosphotyrosine antibody (compare lanes 1 and 2 with lanes 3 and 4). The induced tyrosine

phosphorylation of SHC is largely unaffected by PP1 treatment, bearing witness to the selectivity of the inhibitor for a different kinase. This experiment suggests that another tyrosine kinase mediates the tyrosine phosphorylation of SIRP α, and that this may be a src-family cytosolic tyrosine kinase. Identical results to those shown in Figure 10 were obtained when PDGF-, FGF-, or insulin stimulation was employed in place of EGF stimulation (results not shown).

Although often effective, most inhibitors do not show an absolute specificity for their targets, so further experiments were necessary to test whether or not EGF-induced SIRP α tyrosine phosphorylation was src-family kinase dependent. COS-7 transformed kidney epithelial cells were chosen because they can be effectively transfected. Stable polyclonal cell lines were produced that overexpressed human SIRP α1, which were transiently super-transfected with constructs that overexpressed kinase-inactive forms of different tyrosine kinases. Figure 11 shows the result of this experiment.

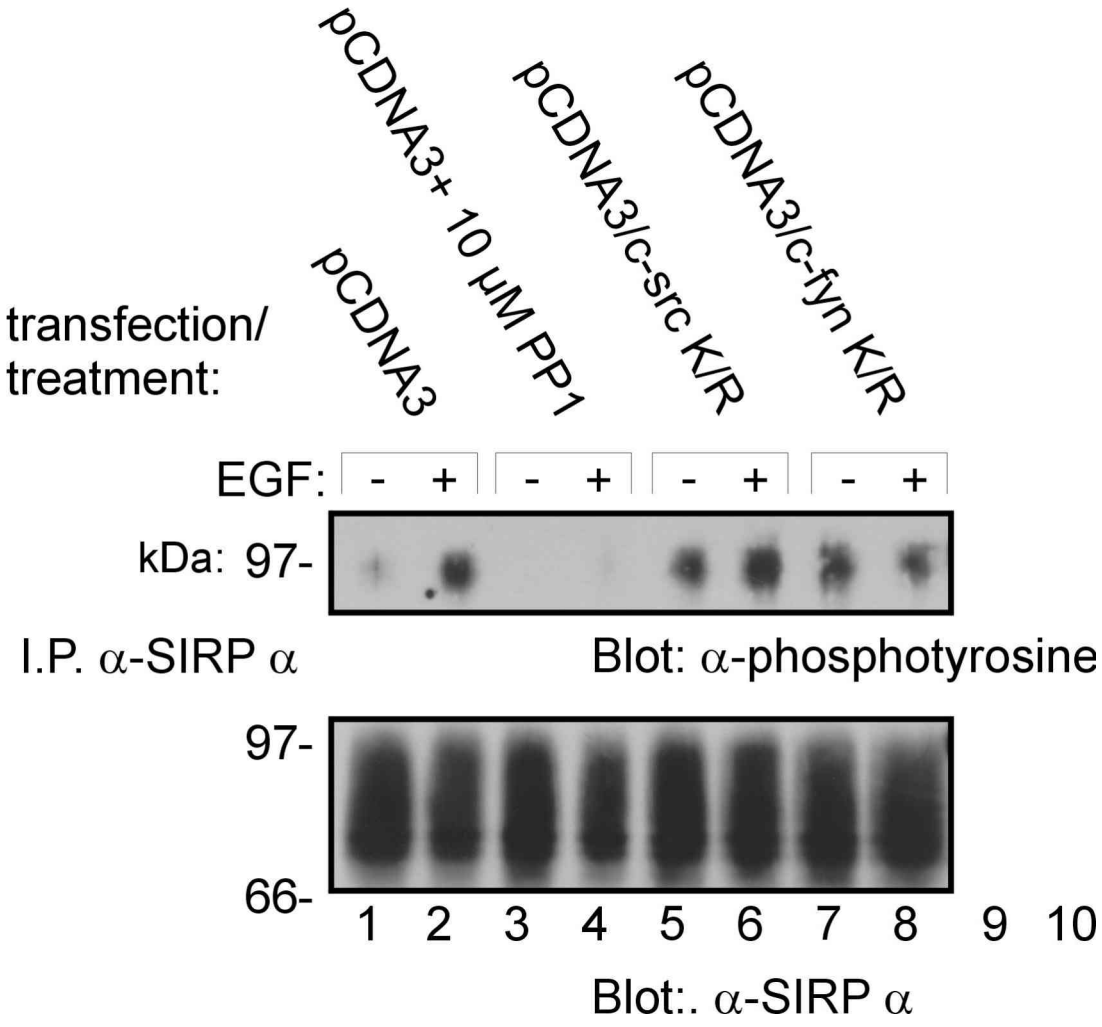


Figure 11 Constitutive tyrosine phosphorylation of SIRP α by overexpression of kinase-inactive mutants of different src-family tyrosine kinases. COS7/SIRP α1 cells transiently transfected with pCDNA3 constructs overexpressing either a kinase inactive form of c-src (pCDNA3/c-src K/R) or c-fyn (pCDNA3/c-fyn K/R). Cells were stimulated with 20 ng/ml EGF for 5 minutes. Cell lysates were immunoprecipitated with ExC_2 anti-SIRP α polyclonal antiserum and immunocomplexes were separated on 10% SDS-PAGE. Tyrosine phosphorylated proteins were detected in Western blot with anti-phosphotyrosine antibodies (upper panel) or ExC_2 antibodies (lower panel).

Neither a kinase-inactive c-src or kinase inactive c-fyn were able to inhibit EGF-induced tyrosine phosphorylation of SIRP α1. Indeed, overexpression of either kinase inactive molecule enhanced the basal tyrosine phosphorylation of SIRP α. This probably means that the kinase activity of these src-family members is not essential for the tyrosine phosphorylation of SIRP α. The enhanced basal tyrosine phosphorylation of SIRP α may therefore be due to a second function of the src-

family tyrosine kinases, e.g. an adaptor role. If the src-family member is able to recruit further kinases to the SIRP α complex through other domains or motifs, it may be indirectly responsible for the effect seen in the experiment.

This all suggests that the sequence of events leading to the tyrosine phosphorylation is a complex program, involving further molecules and possibly another order of tyrosine kinase activity. SIRP α1 indeed associates with molecules that have tyrosine kinase activity in a manner both dependently and independently of its own state of tyrosine phosphorylation (Figure 18, later).

5.3 Association of SIRP β1 with DAP12

5.3.1 SIRP β, DAP12 and Syk form a physical complex in HEK293 cells

To address the relevance of SIRP β as a signal transduction molecule, it was necessary to rationalise a manner by which it would be able to transmit an intracellular signal. SIRP β molecules have no intracellular domain themselves; therefore a logical rationale would be to deduce potential molecules with intracellular domains that might associate with either the extracellular and/or transmembrane domain of SIRP β. KIR2DS2, a member of the Killer Inhibitory Receptor Family (KIR), has been shown to associate non-covalently with a transmembrane adaptor protein (DAP12) involving an ionic interaction between two amino acids of opposing charge within their adjacent transmembrane domains (Figure 12).

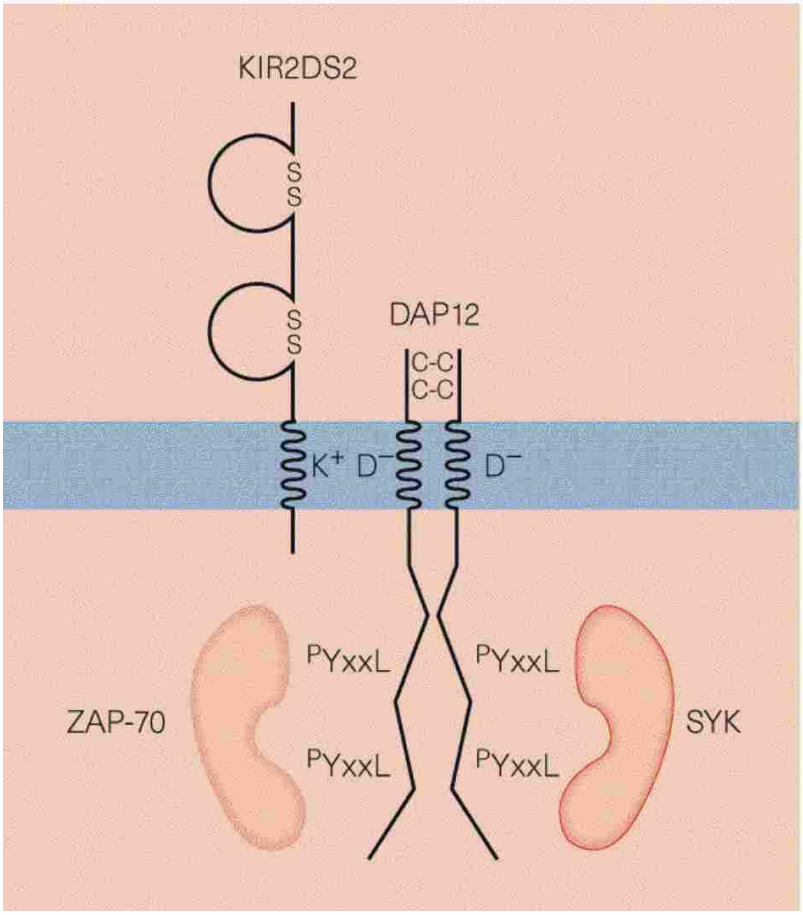


Figure 12 Association of KIR2DS2 with DAP12 (after Colonna, Nature 391, 1998 [67])

Although distinct in terms of primary amino acid sequence from known SIRP family members, KIR2DS2 shares certain structural characteristics with SIRP β. Both are transmembrane glycoproteins with large extracellular and no intracellular domains, and both have a single positively charged amino acid (a lysine K) within the transmembrane of the molecule. It was therefore reasonable to suggest that SIRP β1 might associate with DAP12. To test this hypothesis, HEK293 cells were cotransfected with pCDNA3 overexpressing SIRP β1 and a Flag-tagged DAP12 The result is presented in Figure 13(A).

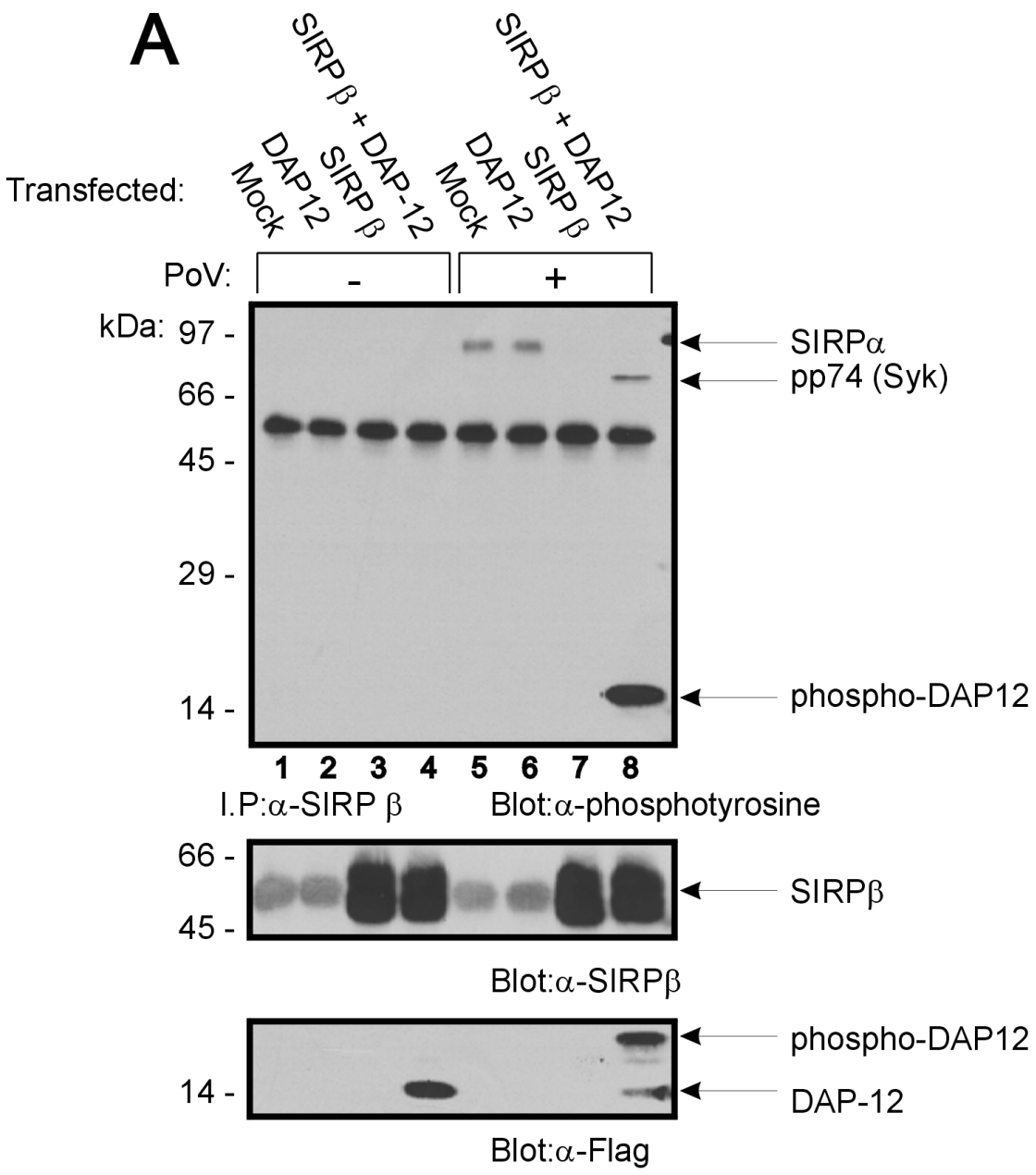


Figure 13(A) Western blots of SIRP β immunoprecipitates from HEK293 cells. Cells have been transiently transfected with pCDNA3 overexpressing either DAP12 ('Flag'-tagged, lanes 2,4,6,8) or SIRP β (lanes 3,4,7,8). Cells were stimulated with 1mM sodium pervanadate for 10 minutes (PoV, lanes 5-8), before lysis. Immunocomplexes were separated by 8%-16% SDS-PAGE. Proteins were detected in Western blot using anti-phosphotyrosine, α-SIRP β or α-Flag antibodies.

Figure 13(A) shows that DAP12 is part of an immunocomplex immunoprecipitated by anti-SIRP β independently of DAP12 tyrosine phosphorylation and is therefore a constitutive association (lower picture, lanes 4 and 8). Accordingly, SIRP β1 is detectable in the immunocomplex immunoprecipitated by anti-Flag antibodies.

Remarkably, a tyrosine-phosphorylated band of 74 kDa is part of the immunocomplex (upper picture, lane 8) following treatment with 1mM sodium pervanadate (PoV). This molecule is identified as the cytosolic tyrosine kinase Syk in the experiment shown in Figure 14. The two bands labelled in Figure 13(A) as SIRP α are probably tyrosine phosphorylated endogenous SIRP α cross-reacting with the anti-SIRP β antibodies. The SIRP α bands are not part of the immunocomplex because they do not precipitate with anti-Flag antibodies and are competed by overexpression of SIRP β1 (compare lanes 5&6 with Lanes 7&8).

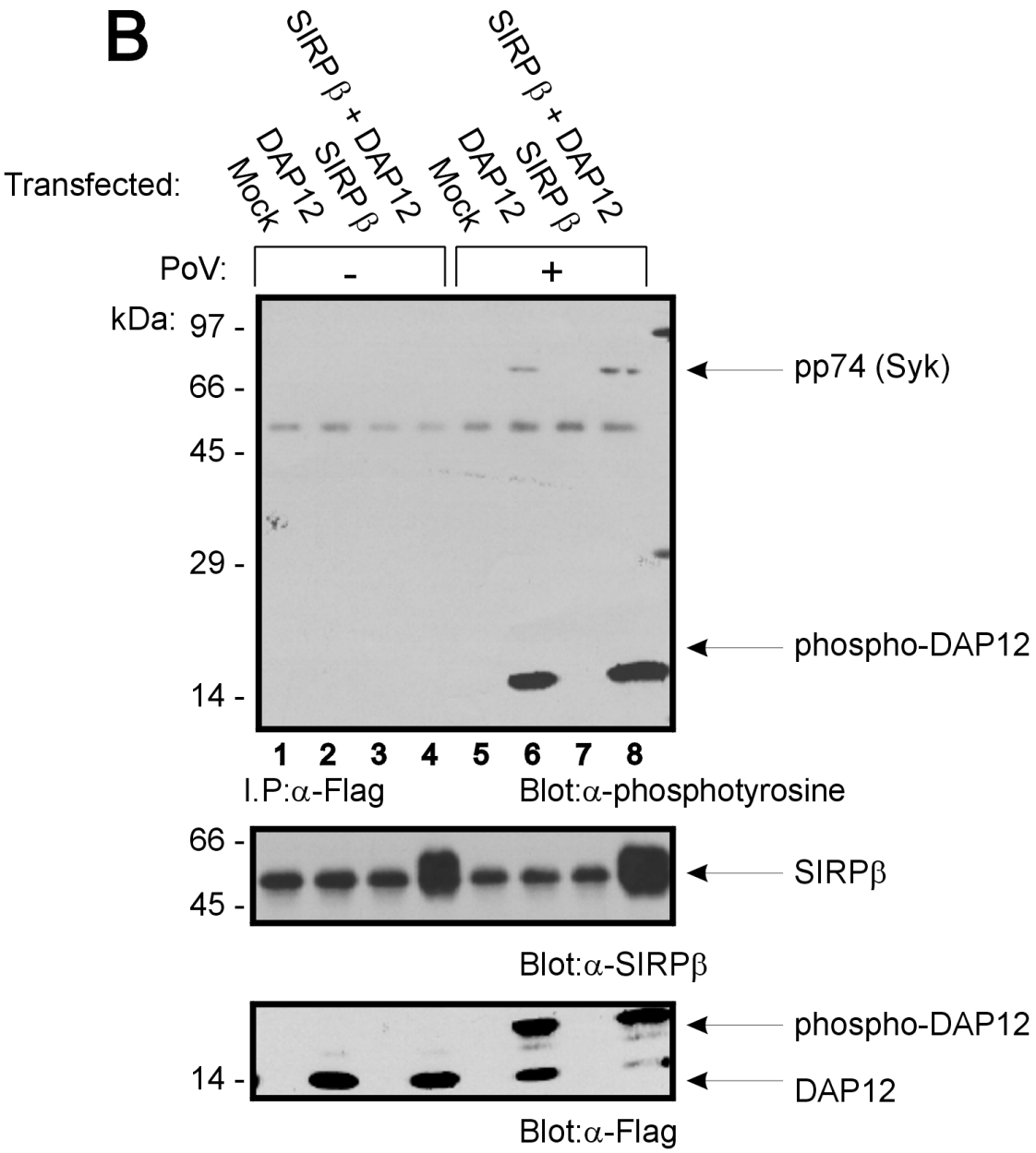


Figure 13(B) Western blots of DAP12 (Flag) immunoprecipitates from HEK293 cells. Cells have been transiently transfected with pCDNA3 overexpressing either DAP12 ('Flag'-tagged, lanes 2,4,6,8) or SIRP β (lanes 3,4,7,8). Cells were stimulated with 1mM sodium pervanadate for 10 minutes (PoV, lanes 5-8), before lysis. Immunocomplexes were separated by 8%-16% SDS-PAGE. Proteins were detected in Western blot using anti-phosphotyrosine, α-SIRP β or α-Flag antibodies.

The upper picture in Figure 13(B) shows that pp74 (Syk) associates with anti-Flag immunoprecipitates and overexpression of SIRP β1 neither interferes with, nor promotes this association. It is assumed therefore that Syk associates with

SIRP β through DAP12 and not *vice versa*, and that the associations utilise independent binding sites on DAP12.

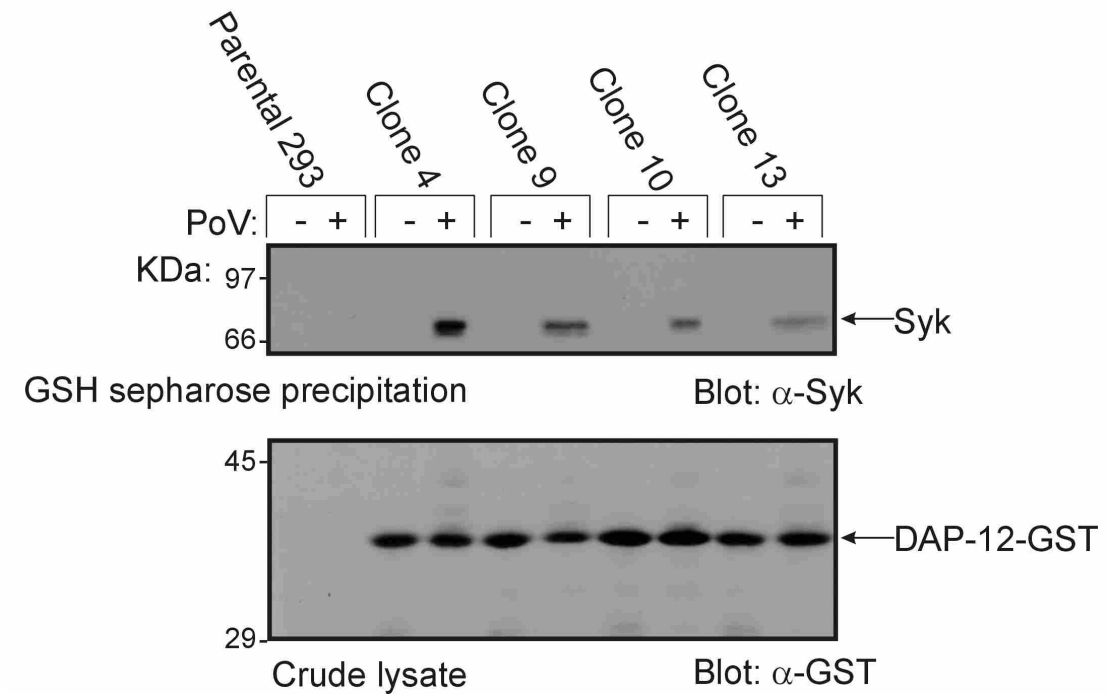


Figure 14 Identification of pp74 as Syk. Comparison of parental HEK293 cells with four selected clones from a panel of stable G418-selected monoclonal cell lines produced from HEK293 cells transfected with pSj26/DAP12. Cells were treated with 1mM sodium pervanadate (PoV) for 10 minutes and GST-DAP12 was precipitated from the cell lysates with GSH sepharose. Complexes were separated on 8% - 16% SDS PAGE and bands were visualised in Western blot with anti-Syk polyclonal antiserum, or anti GST polyclonal antiserum as control.

The experiment presented in Figure 14 identifies pp74, the kinase that directly associates with DAP12 as Syk.

5.3.2 Syk expression in non-haematopoietic cells

Since Syk is thought to be a tyrosine kinase restricted to haematopoietic cell lineages, it is necessary to confirm the presence of endogenous Syk in HEK293 cells. PCR amplification of Syk from cDNA prepared from HEK293 cells confirms this using primers specific for the coding region of human Syk. (Figure 15, lanes 1-3).

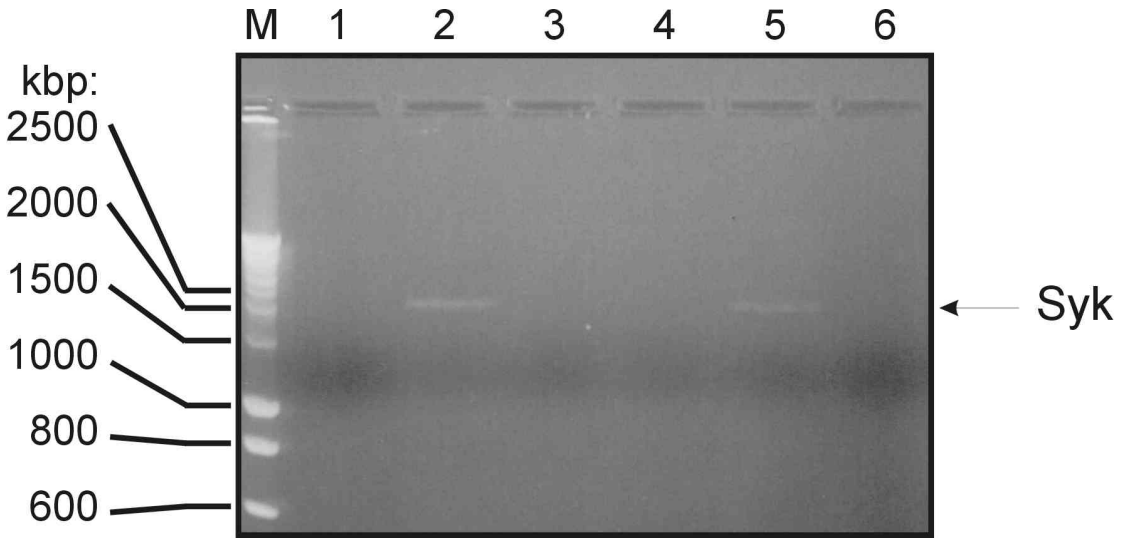


Figure 15 Amplification of Syk from HEK293 and NIH3T3. PCR from cDNA prepared from HEK293 and NIH3T3 cells using PCR primers specific for human (lanes 1-3) and murine (lanes 4-6) Syk, respectively. Lanes 1,3-4,6 with no template cDNA as control, Lane 2 contains HEK293 cDNA, Lane 5 contains NIH3T3 cDNA. 30 cycles of PCR were performed.

The result in Figure 15 shows a single band of the expected size amplified from HEK293 confirming the endogenous expression of Syk in these cells and outside the haematopoietically-derived lineages. The analogous experiment is presented to show expression of murine Syk in NIH3T3 cells (4-6) which then provides an ideal model adherent cell line for the further biological experiments that are presented later.

The previous experiments support the existence of a potential multiprotein complex composed of SIRP β, DAP12 and Syk. The *in vitro* kinase assay presented in Figure 16 provides further evidence of a link between this complex and proteins that may be involved in downstream signal transduction pathways.

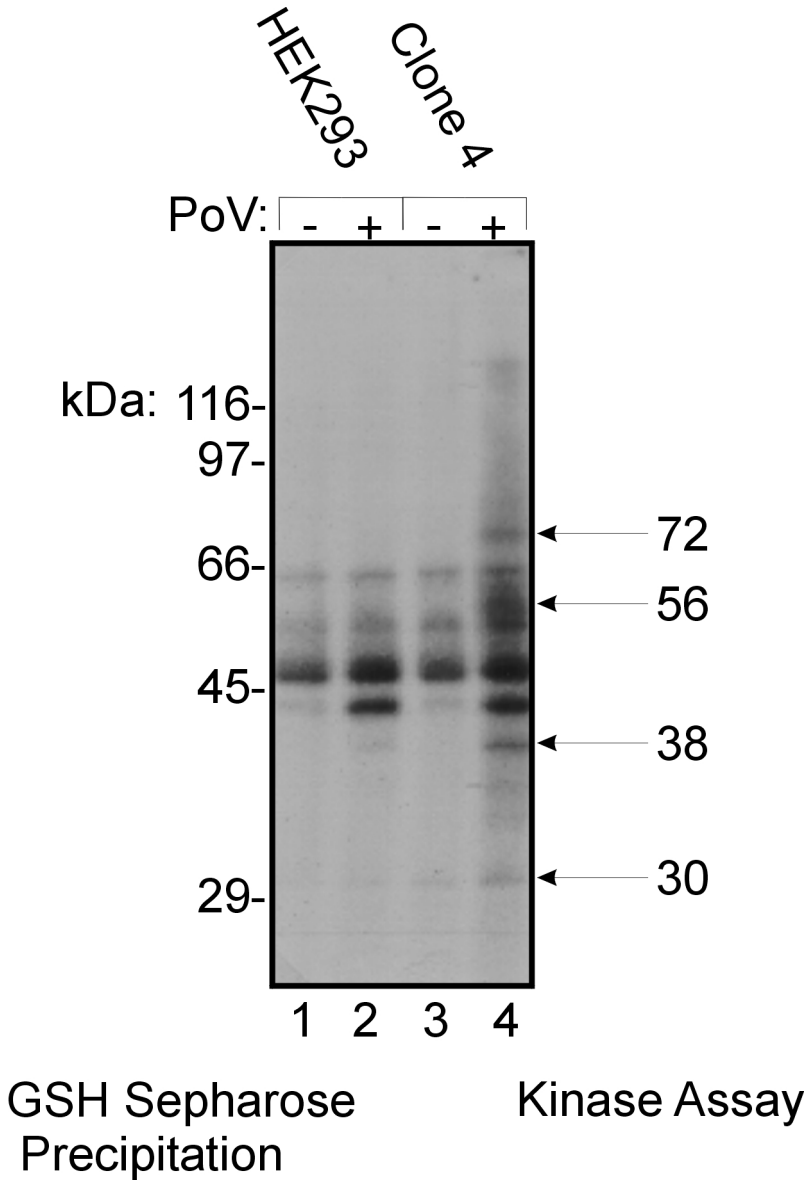


Figure 16 Evidence of a multiprotein complex clustered around GST-DAP12 in activated HEK293 cells. Parental HEK293 cells (lanes 1-2) and HEK293/GST-DAP12 clone 4 (lanes 3-4), were obtained as outlined in Figure 14. Cells were treated with 1mM sodium pervanadate (PoV) for 10 minutes (lanes 2 and 4) and GST-DAP12 was precipitated from the cell lysates with GSH sepharose. An *in vitro* kinase assay was performed on the complexes. No exogenous substrate was added. The complexes were separated on 8% -16% SDS PAGE and bands were visualised by autoradiography.

The *in vitro* kinase assay presented in Figure 16 reveals a number of phosphorylated bands associated in a complex coprecipitating with GST-DAP12, one of these (at ~72 kDa) is probably Syk. Two other outstanding bands of 38- and 30 kDa are also visible. It is possible that p38 could be the MAP kinase of the

same name, since its association with Syk in haematopoietic lines has already been reported. The 56 kDa band may correspond to a src-family tyrosine kinase, since these are required for full activation of Syk in the context of the B-cell receptor. Although none of these bands other than Syk were strong enough to be detected with specific antibodies in Western blot, this experiment strongly suggests the formation of a multiprotein tyrosine phosphorylated signal transduction complex assembling around DAP12 in activated cells.

5.3.3 SIRP β/DAP12 complex inhibition by SIRP α coexpression

SIRP α is an ITIM motif-containing protein, and the negative influence of ITIM-containing inhibitory receptors upon the ITAM-containing activating receptors is increasingly often observed in immunology[2].

Since DAP12 is an ITAM-containing receptor, the potential of overexpressed SIRP α1 to influence the signalling through SIRP β/DAP12 was investigated. The first experiment (Figure 17) suggests that SIRP α1 overexpression can inhibit the kinase activity of Syk. At the same time, Syk becomes tyrosine phosphorylated, suggesting that this inhibition may involve a tyrosine kinase activity.

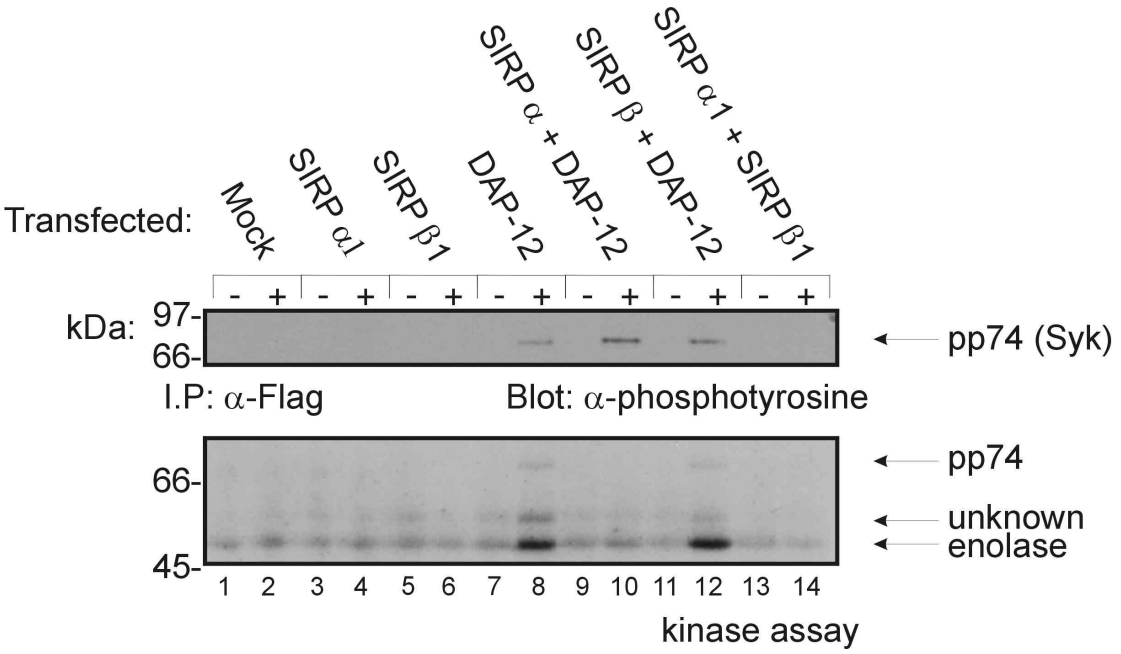


Figure 17 Inhibitory influence of SIRP α on DAP12 signalling. HEK293 cells were transfected with pCDNA3/DAP12 (lanes 7,8,11,12) along with pCDNA3/SIRP α1 (lanes 3-4,9-10,13-14) and pCDNA3/SIRP β1 (lanes 5-6, 11-14). Cells were stimulated with 1mM sodium pervanadate (PoV) as indicated. DAP12 was immunoprecipitated from the cell lysates and an *in vitro* kinase assay was performed using enolase from rabbit muscle as exogenous substrate. Proteins were separated on 12% SDS-PAGE and tyrosine phosphorylated proteins were detected in Western blot (upper panel). Proteins that had been phosphorylated during the kinase assay were detected by autoradiography (lower panel).

Figure 17 shows that DAP12 coprecipitates with a kinase activity that is able to phosphorylate enolase as well as two other proteins. Enolase is a popular substrate used in *in vitro* kinase assays to detect src family kinase activity. One of these phosphorylated proteins is the exact same size as Syk while the other approximately 55 kDa protein is unknown. It may correspond to CSK, a 55kDa SH-2 domain-containing protein that has been shown to associate *in vitro* with SIRP α[20]. A single tyrosine phosphorylated protein of 74 kDa again appeared in the anti-phosphotyrosine Western blot. This means that either pp55 or pp74 or both possess a kinase activity. Cotransfection of SIRP α1, however, abolishes the radioactive bands in the kinase assay (compare lane 8 with lane 10) and therefore the coprecipitating kinase activity. Interestingly, although the kinase activity has been inhibited by SIRP α1 coexpression, pp74 remains an associated tyrosine phosphorylated protein and an increased intensity of signal and gel mobility shift

suggests that it may be even more highly tyrosine phosphorylated, and that a kinase my be involved in this inhibition. The next experiment looked to see whether SIRP α1 coprecipitated with kinase activity.

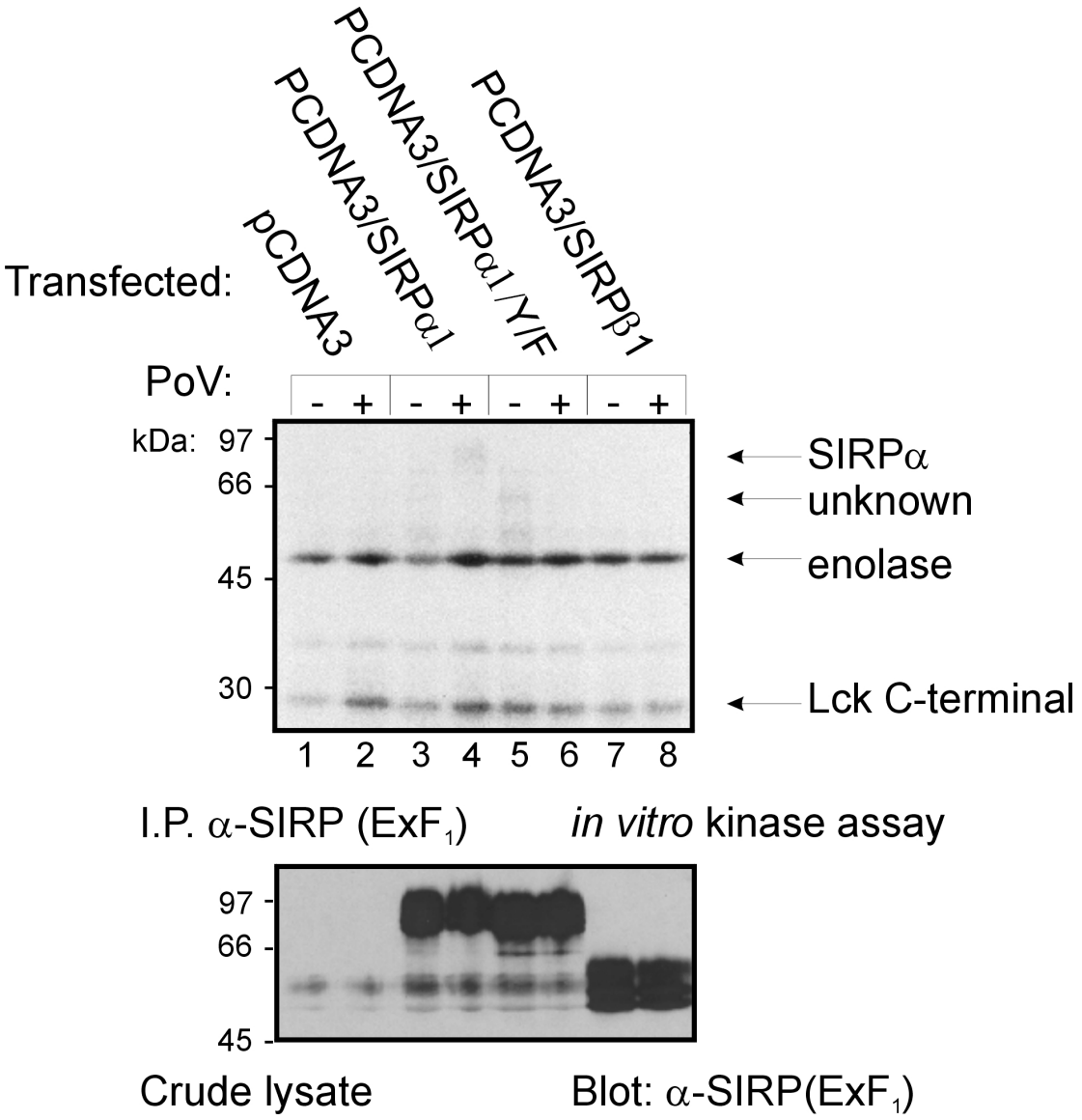


Figure 18 Kinase activity associated to SIRP α1. HEK293 cells were transfected with pCDNA3/SIRP α1 (lanes 3 and 4), pCDNA3/SIRP α1 Y/F (lanes 5 and 6) or pCDNA3/SIRP β1 (lanes 7 and 8). Cells were treated with 1mM sodium pervanadate (PoV) for 10 minutes, as indicated. SIRP proteins were immunoprecipitated from the lysates with ExF_1 antiserum that recognises both human SIRP α and SIRP β [1] and an *in vitro* kinase assay was performed using both enolase and a GST fusion protein of the C-terminal of the src-family kinase Lck that contains the Y527 inhibitory tyrosine.

Treatment of cells with 1mM sodium pervanadate (PoV) leads to the activation of at least two kinase activities present in the complex immunoprecipitated by anti SIRP antibodies (Figure 18). Enolase, as well as a GST fusion protein of the C-terminal of src-family tyrosine kinase Lck[68] were used as exogenous substrates.

The C-terminal portion of Lck contains the inhibitory tyrosine (Y527 in c-src) that inhibits src kinase activity through an intramolecular association with the SH2-domain when phosphorylated. The phosphorylation of this Lck-GST fusion protein is therefore an indication of a kinase activity present that may be able to inhibit src-family tyrosine kinases. The activity that is able to phosphorylate the inhibitory tyrosine in the C-terminal of Lck, is dependent upon tyrosine phosphorylation of SIRP α because there is no inducible effect when SIRP α1 Y/F is overexpressed (compare lanes 3 and 4 with lanes 5 and 6). A second kinase activity can inducibly phosphorylate enolase, but this is also the case when SIRP α1 Y/F is overexpressed, suggesting that it associates to SIRP α independently of the ITIMs in SIRP α.

5.3.4 Stimulation of SIRP β1 complex signalling with mAb B1D5C3

Since SIRP β1 has no known ligand, it was clear that it would be useful to obtain an activating SIRP β1-binding antibody that could be added to cells to activate the SIRP β1 complexes on the surface by coligation. This would enable the examination of SIRP β/DAP12 signalling by a more precise method of stimulation. Empirical results with the self-produced α-SIRP β ex antibody were not forthcoming (results not shown), therefore, SIRP α1exGST was used in collaboration with H-J Buehring and M. Seiffert to produce SIRP β1-selective monoclonal antibodies (mAbs) that may be used in the stimulation experiments described above. Figure 19 illustrates the specificity of B1D5C3, one such mAb that was isolated that successfully immunoprecipitated overexpressed SIRP β1 but not SIRP α1in HEK293 cells.

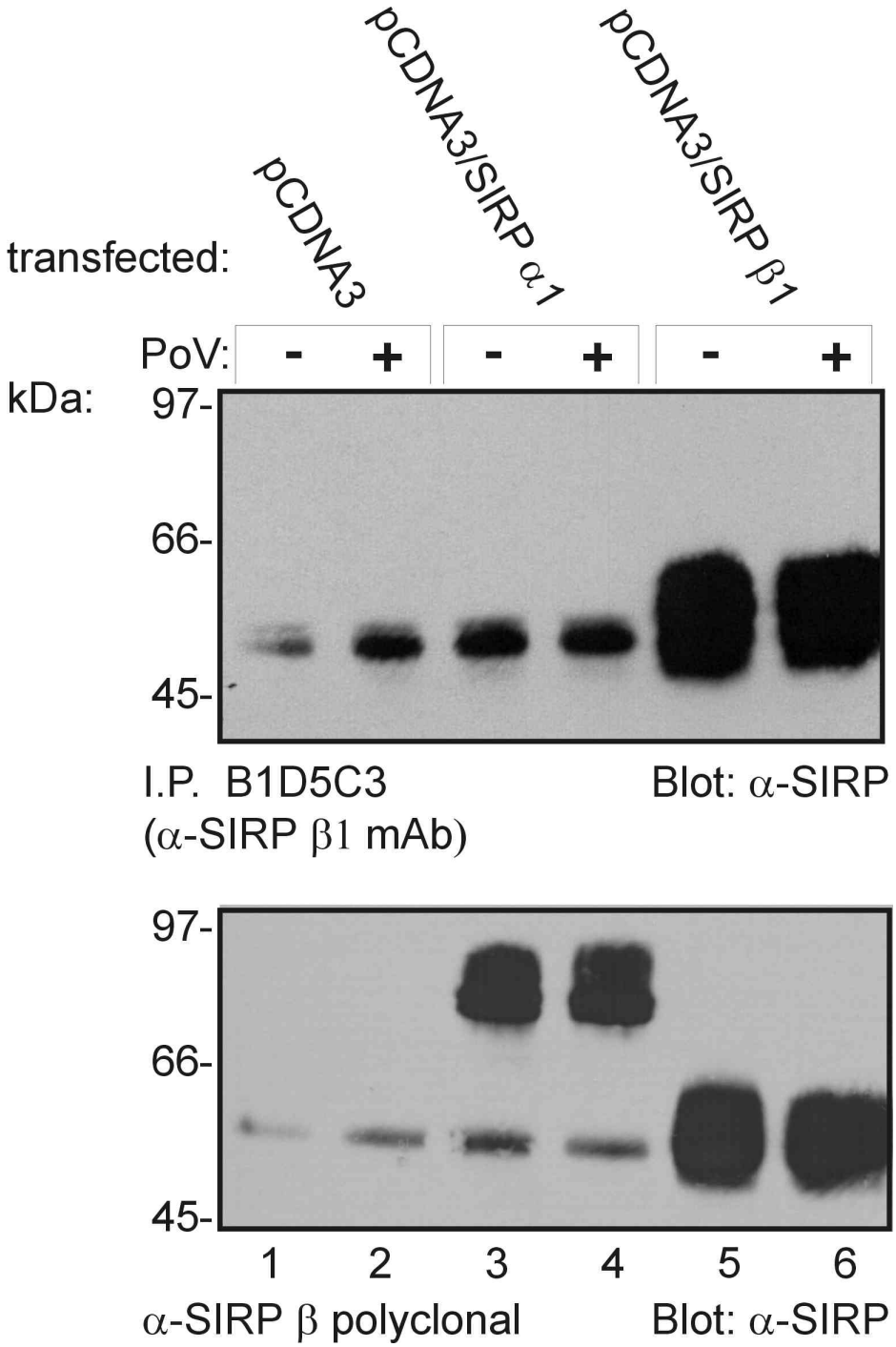


Figure 19 Selectivity of mAb B1D5C3 for SIRP β over SIRP α. HEK293 cells were transiently transfected with pCDNA3 (lanes 1-2), pCDNA3/SIRP α1 (lanes 3-4) or pCDNA3/SIRP β1 (lanes 5-6). Cells were stimulated with 1mM sodium pervanadate (PoV) for 10 minutes. Lysates were immunoprecipitated with mAb B1D5C3 (upper panel) or ExC2 polyclonal antiserum (lower panel). Immunocomplexes were separated on 10% SDS PAGE and proteins were detected in Western blot with a mixture of SIRP α and SIRP β antisera (α-SIRP).

The experiment presented in Figure 19 shows that while the α-SIRP β ex antiserum cannot selectively immunoprecipitate SIRP β1 in favour of SIRP α1, mAb B1D5C3 recognises SIRP β1 but not SIRP α1. Therefore mAb B1D5C3 is indeed selective for SIRP β1. This mAb was tested in subsequent experiments to assess its potential as an activating mAb.

To reveal possible tyrosine phosphorylation of SIRP β–associated components via coligation of the SIRP β/DAP12 complex, mAb B1D5C3 was added to HEK293/GST-DAP12 cells overexpressing SIRP β1 over a time course varying from 2 minutes to 1 hour. The results were analysed by Western blotting with anti-phosphotyrosine antibodies and are shown in Figure 20.

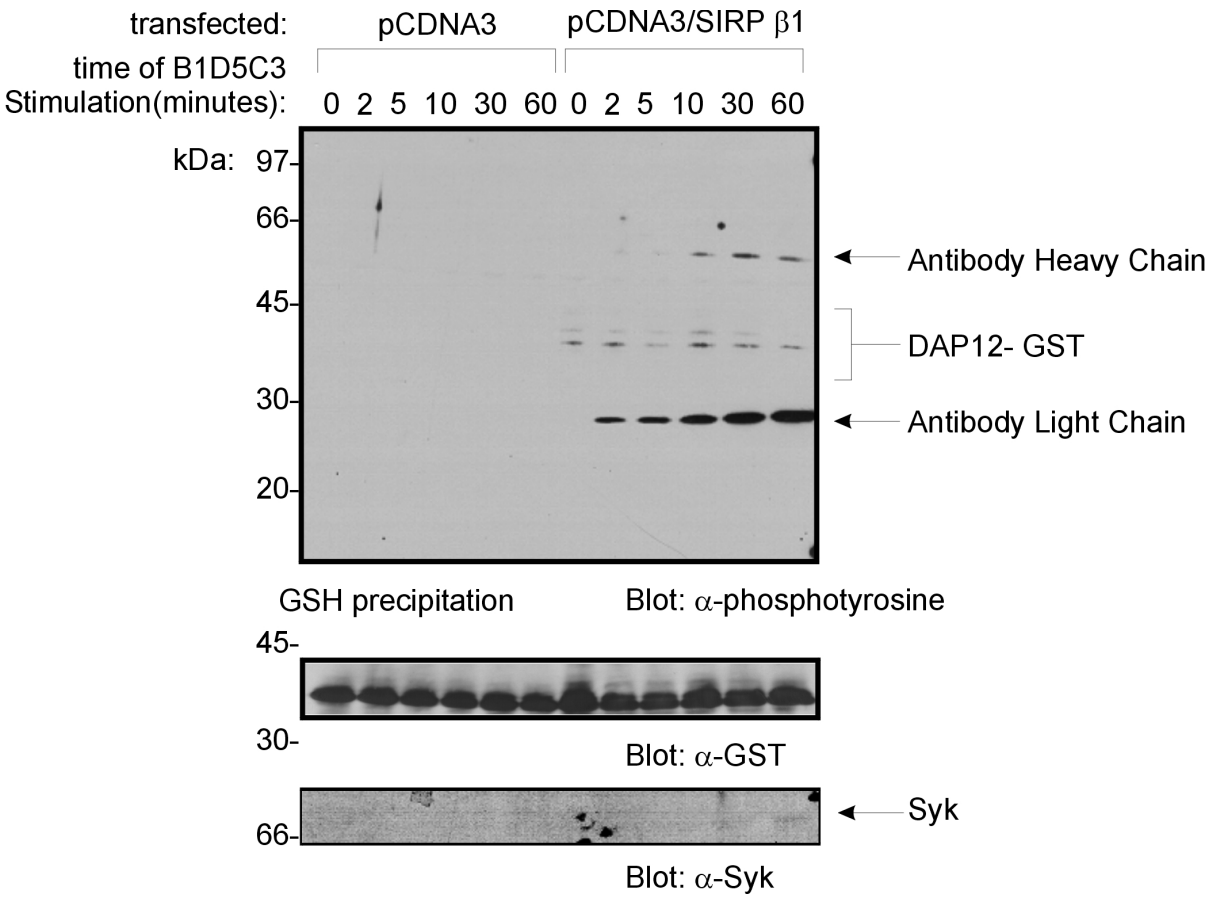


Figure 20 Coligation of SIRP β1/DAP12 complex with mAb mAb B1D5C3. HEK293/GST-DAP12 cells were transiently transfected with pcDNA3 or pCDNA3/SIRP β1, as indicated. Cells were stimulated with 20 µl dialysed B1D5C3 hybridoma supernatants for the times indicated and the cells were washed twice in PBS before lysis. Lysates were precipitated with GSH sepharose and the complexes were separated on 8%-16% SDS PAGE. Tyrosine phosphorylated proteins were detected in Western blot with anti-phosphotyrosine antibodies.

Figure 20 shows that mAb B1D5C3 is able to bind to SIRP β1. The two bands of ~28 and ~55 kDa (latter half of the Figure) correspond to the light and heavy chains of mAb B1D5C3 as they are recognised by the secondary anti-mouse antibody used in the Western blot. These bands increase in intensity with time, and require that SIRP β1 is overexpressed to bind to the cells and come down in the complex coprecipitated with the GST fusion protein. The two bands of ~38 and ~40 kDa could be identified as GST-DAP12 by western blotting with α-GST antibody (middle panel) [69]. These bands are only visible where SIRP β is overexpressed, but also where there is no stimulation by antibody. This suggests

that overexpression of SIRP β1 alone has an effect upon signalling through DAP12.

Although mAb B1D5C3 stimulation is weak, at 1 hour Syk is visible as a weak band in western blot (lower panel). This suggests that the antibody does have an effect on signalling through the SIRP β/DAP12 complex.

SIRP α may recruit tyrosine kinases to the SIRP β/DAP12 complex thereby inhibiting its activity (Figures 17 and 18). To identify kinases that could modify GST-DAP12 tyrosine phosphorylation as part of a SIRP α complex, kinase inactive molecules were overexpressed with SIRP α1 in GST-DAP12 cells transiently overexpressing SIRP β1. Kinase-inactive CSK and PYK2 were chosen because they have both been shown to bind to SIRP α[20 23]. Treatment with mAb B1D5C3 was repeated in case an effect could be seen together with this overexpression.

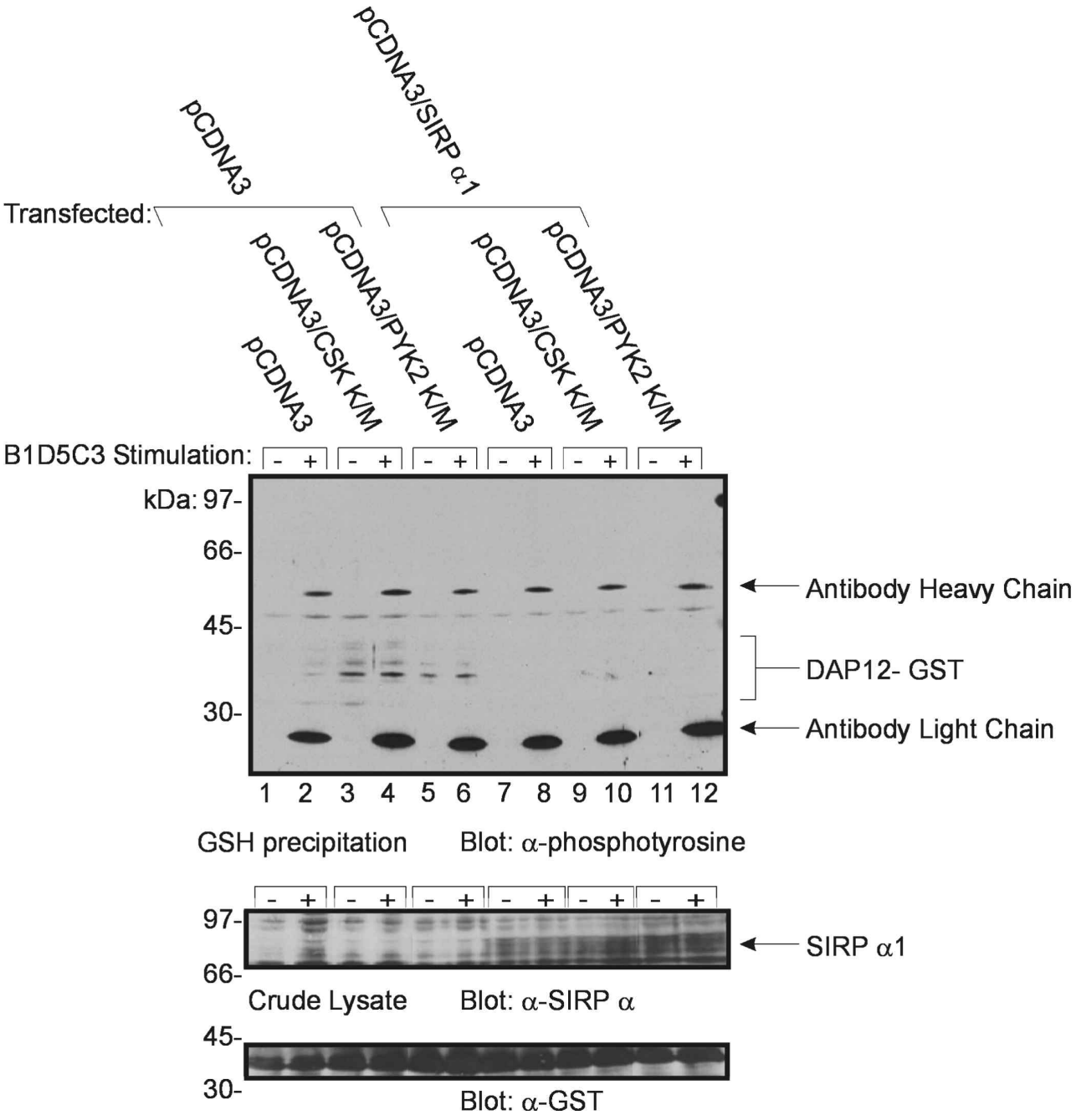


Figure 21 Enhancement of constitutive GST-DAP12 tyrosine phosphorylation by exposure to kinase inactive molecules. HEK293/GST-DAP12 cells were transiently transfected with SIRP β1 (all lanes) together with pCDNA3/SIRP α1 (lanes 7-12) and pCDNA3/CSK K/M (lanes 3-4, 9-10) or pCDNA3/PYK2 K/M (lanes 5-6, 11-12). Cells were stimulated with 20 µl dialysed B1D5C3 hybridoma supernatants for 30 minutes (even lanes). Lysates were precipitated with GSH sepharose and the complexes were separated on 8%-16% SDS PAGE. Tyrosine phosphorylated proteins were detected in Western blot with anti-phosphotyrosine antibodies.

The experiment shown in Figure 21 suggests that both a kinase inactive CSK (CSK K/M) and, to a lesser extent a kinase inactive PYK2 (PYK2 K/M) enhance the basal tyrosine phosphorylation of GST-DAP12. This tyrosine phosphorylation is probably independent of treatment with mAb mAb B1D5C3, and is also independent of SIRP α1 overexpression. Overexpression of SIRP α1, on the other hand inhibits the tyrosine phosphorylation of GST-DAP12. This is reminiscent of the experiment (Figure 17) where overexpression of SIRP α1 inhibits the kinase activity of Syk but enhances its tyrosine phosphorylation. Nevertheless co-overexpression of either CSK K/M or PYK2 K/M does not influence this. Therefore neither of these kinases can be implicated in GST-DAP12 phosphorylation from this experiment.

Because mAb B1D5C3 may therefore not be effective as a tool to elicit signalling through SIRP β/DAP12 in HEK293 cells, these experiments were discontinued.

5.4 Cellular Localisation of SIRP β and DAP12

Overexpression of SIRP β1 in GST-DAP12 cells (Figure 20) induces phosphorylation of GST-DAP12. This may be due to an influence upon the subcellular localisation of DAP12 by SIRP β1. To test this hypothesis, the localisation of overexpressed GFP fusion proteins of SIRP β1 (SIRP β1-GFP, Figure 22) and DAP12 (DAP12-GFP, Figure 23) were examined in NIH3T3 cells. To compare the influence of SIRP β1 on localisation of DAP12, overexpressed DAP12-GFP was compared with and without overexpressed SIRP β1 (Figure 23). Images representative of typical positive transfectants are presented below.

5.4.1 Subcellular localisation of SIRP β1-GFP

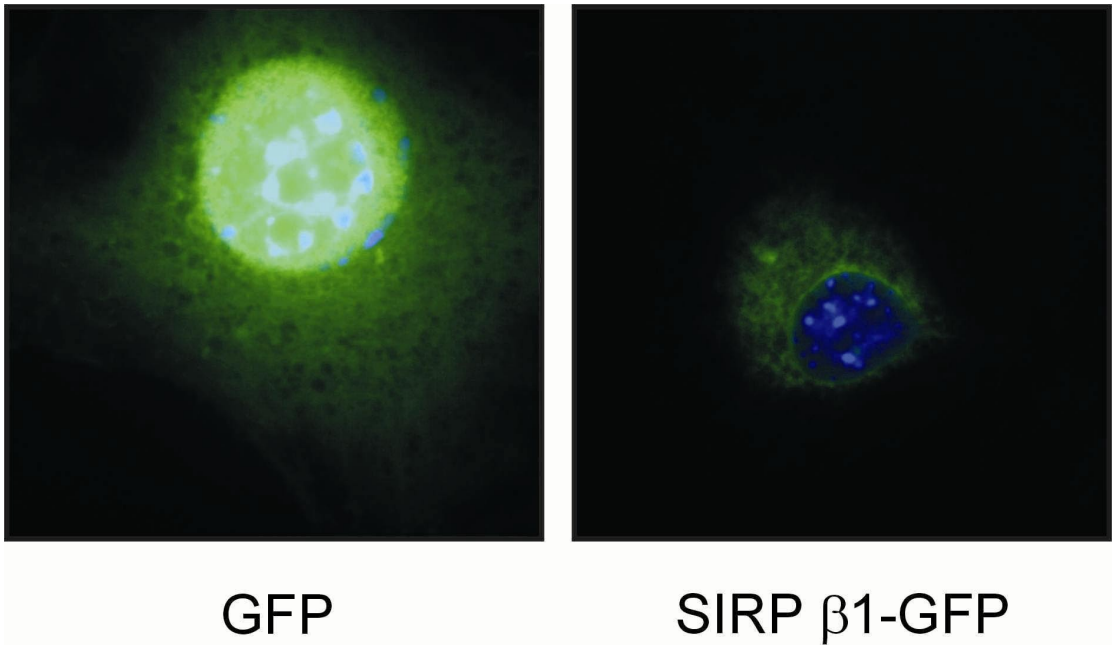


Figure 22 Representation of pCDNA3/SIRP β1-GFP expression in NIH3T3 cells. NIH3T3 cells transiently transfected with pCDNA3/GFP vector expressing either GFP alone (left panel) or a SIRP β1-GFP fusion protein (right panel).). Cells were fixed in paraformaldehyde and nuclei were stained with DAPI (blue) (DAPI: λ_{ex}=358 nm, λ_{em}=461nm). Protein expression (green) was visualised by fluorescence microscopy (GFP λ_{ex}= 495 nm, λ_{em}=525 nm).

When cells were transfected with pCDNA3/ SIRP β1-GFP, there were far fewer cells that visibly expressed a GFP-fusion protein than when GFP was expressed alone. The distribution of SIRP β1-GFP is perinuclear, and granular in distribution, suggesting a localisation in the endoplasmic reticulum. Little SIRP β1 was present in the cell membrane.

5.4.2 Comparison of localisation of DAP12-GFP with and without SIRP β1

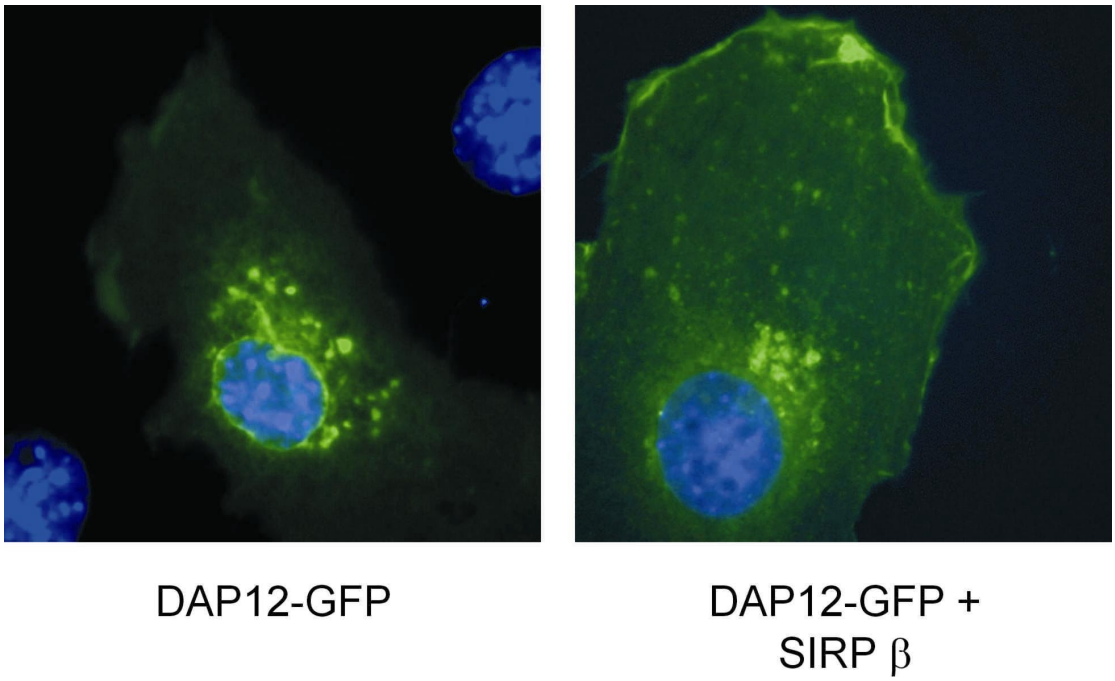


Figure 23 Translocation of DAP12 to the cell membrane after SIRP β1 coexpression. The picture compares NIH3T3 cells transiently transfected with pCDNA3/DAP12-GFP either alone (left) or pCDNA3/DAP12-GFP cotransfected with pCDNA3/SIRP β1 (right). Cells were prepared and analysed as described in Figure 22.

Cells expressing DAP12 following transfection with pCDNA3/DAP12-GFP are also fewer in number than GFP. Where a signal is found, the cellular localisation is also perinuclear, although with a different appearance to that of SIRP β1 (compare Figure 23 with 22). It is known that the coexpression of SIRP β influences the cellular distribution of DAP12, causing DAP12 to migrate to the cell membrane where it has been detected using FACS [70]. This is confirmed by overexpression of SIRP β1 together with DAP12-GFP (Figure 23). The influence on the localisation of DAP12 is visible when the left and right pictures are compared, with significantly more DAP12-GFP localised to the membrane.

5.4.3 DAP12 membrane translocation affects its signalling potential

Experiments with overexpression presented in previous sections have suggested that SIRP β1 influences both the tyrosine phosphorylation and subcellular distribution of DAP12. The following experiment confirms that this translocation to the cell membrane places DAP12 within the proximity of tyrosine kinases. SIRP β1 was overexpressed in DAP12-GST cells, and the cells were treated with sodium pervanadate to inhibit tyrosine phosphatases. To confirm the inhibitory effect of SIRP α molecules upon the SIRP β/DAP12 complex, SIRP α1 was also expressed. Tyrosine phosphorylation of DAP12 was assessed in western blot with anti-phosphotyrosine antibodies.

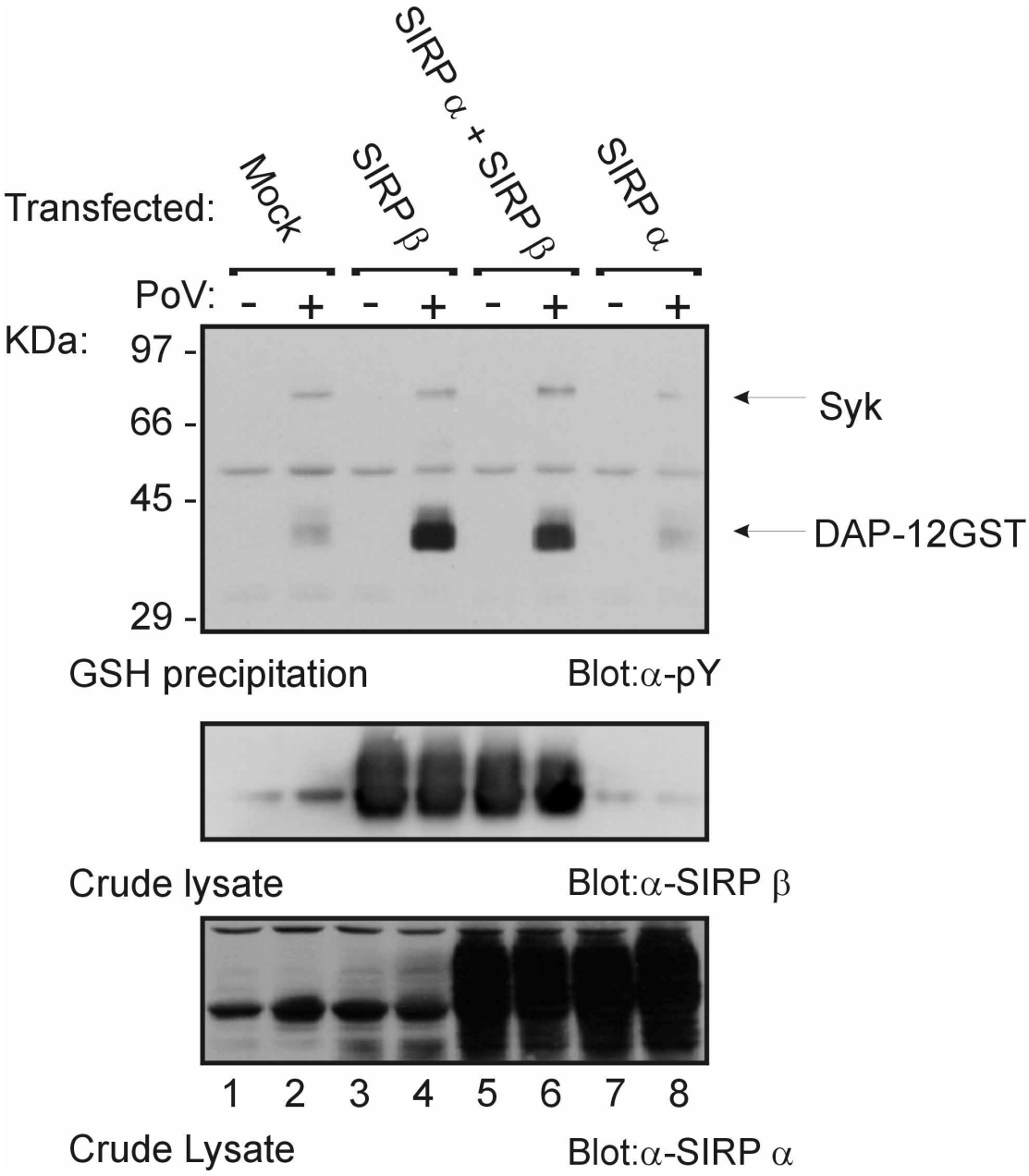


Figure 24 Repositioning of DAP12 at the cell membrane following SIRP β1 overexpression places it within the vicinity of tyrosine kinases. HEK293/DAP12-GST cells transiently overexpressing SIRP β1 (lanes 3-6), and SIRP α1 (lanes 5-8) stimulated with 1mM sodium pervanadate (PoV) for 10 minutes. DAP12-GST was precipitated from cell lysates and proteins were separated on 12% SDS PAGE tyrosine phosphorylated proteins were detected in Western blot with anti-phosphotyrosine antibodies.

When HEK293/DAP12-GST cells are transfected with SIRP β1 and treated with sodium pervanadate, DAP12-GST is far more strongly tyrosine phosphorylated than when SIRP β1 is not overexpressed. When SIRP α is coexpressed with

SIRP β1, there is an inhibition of DAP12-GST tyrosine phosphorylation, although this is not strong enough to reverse the effect of SIRP β1 overexpression. Differences are also seen in tyrosine phosphorylation of the coprecipitating band likely to be Syk (see Figure 14), but it is not possible to tell whether this is due to tyrosine phosphorylation of DAP12-GST (essential for association of Syk) or of the protein itself.

5.4.4 Dependence of DAP12 upon SIRP β1 coexpression and *vice versa*

To address the potential biological consequences of a collaborative signalling complex formed between SIRP β and DAP12, NIH3T3-derived cell lines were prepared stably-expressing either SIRP β or DAP12 or both. To minimise the cytotoxic insult produced by sequential infections and multiple antibiotic selection procedures, a vector was derived from pLXSN that possessed an internal ribosomal entry site (IRES) in the middle of the multiple cloning site (MCS), enabling two flanking ORFs to be cloned (section 5.4.1.1). The vector (termed pIRES) is then able to express two sequences of interest as a bicistronic transcript, resulting in both ORFs being theoretically transcribed in a 1:1 molar ratio. An interesting phenomenon was observed upon testing these constructs in transient transfection in HEK293 cells (Figure 25).

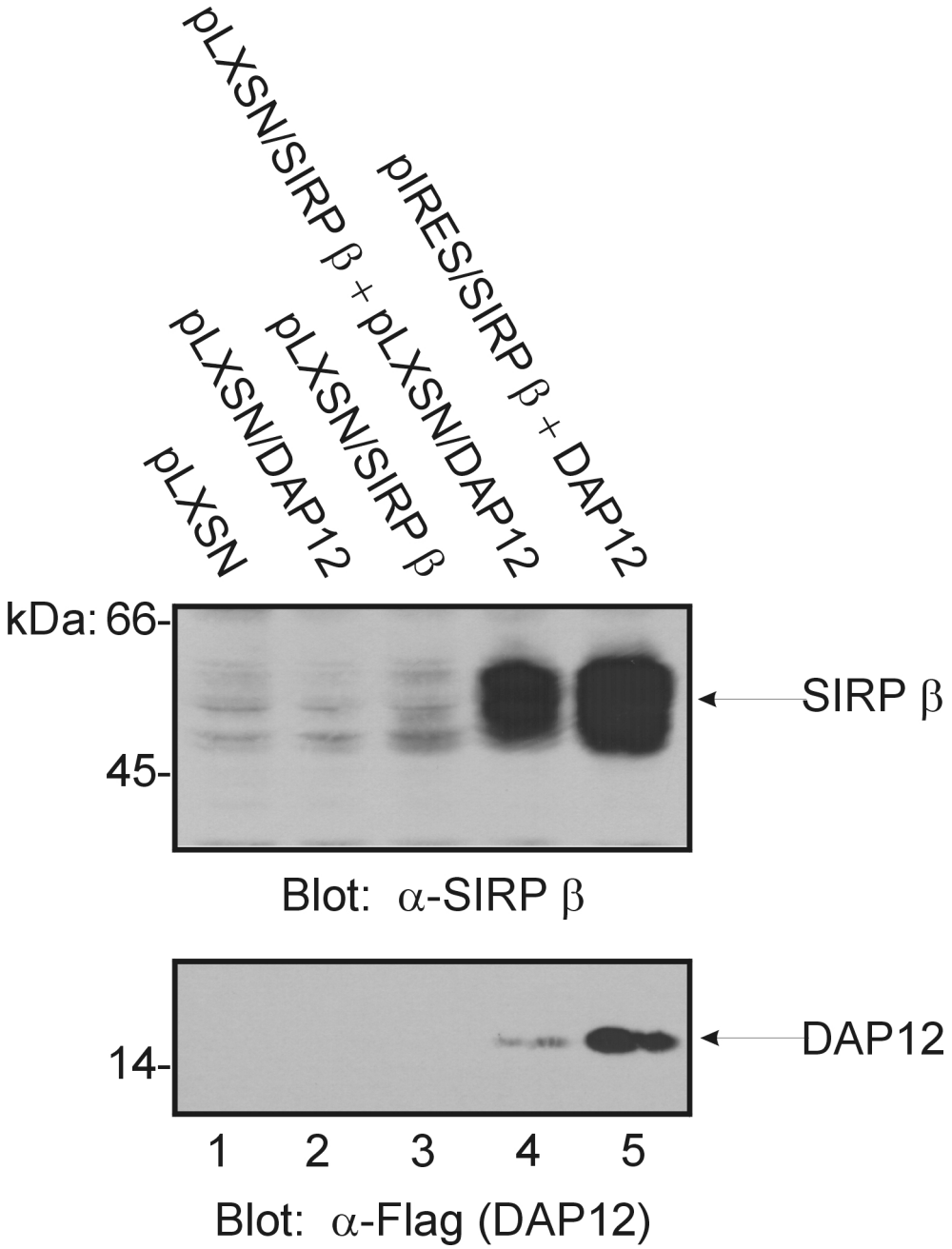


Figure 25 Expression of SIRP β is higher when DAP12 is present, and *vice versa*. HEK293 cells transfected with pLXSN (lane 1), pLXSN/DAP12 (lanes 2 and 4), pLXSN/SIRP β1 (lanes 3 and 4), and pIRES/SIRP β1 + DAP12 (lane 5). Cells were lysed in standard 0.1% Triton X-100 lysis buffer and balanced quantities of protein were loaded onto 8%-16% SDS PAGE. Proteins were detected in Western blot using α-SIRP β antibodies (top panel) and α-Flag antiserum (lower panel).

The experiment shown in Figure 25 shows the surprising result that SIRP β1 or DAP12 are only detectable in Western blot when accompanied by each other. Moreover, signal intensity of both SIRP β1 and DAP12 are increased when the

pIRES vector is used compared to when plasmids with single inserts were cotransfected (compare lanes 4 and 5). This is probably due to efficiency of transfection, i.e. not all cells successfully transfected contain both plasmids. This supports the hypothesis that both proteins are required for the sustained expression of either partner. There is no significant difference in this result when a RIPA buffer is used (not shown) instead of Triton X-100 lysis buffer for cell lysis so it is likely that DAP12 affects the SIRP β signal in Western blot by influencing its presence in the cell and not its subcellular localisation.

5.4.5 SIRP β membrane localisation depends upon DAP12

The influence of SIRP β upon the subcellular localisation of DAP12 has now been addressed, but not how this affects the distribution of SIRP β1 in the cell. At high levels of overexpression, SIRP β1-GFP is perinuclear, probably in the endoplasmic reticulum (Figure 22). If SIRP β escorts DAP12 to the membrane, it is likely that SIRP β1 would also be present in the cell membrane when DAP12 is overexpressed. To assay the subcellular localisation of SIRP β1 at lower levels of overexpression similar to those that produced the effect seen in Figure 25, immunofluorescence with mAb B1D5C3 was necessary (Figure 26).

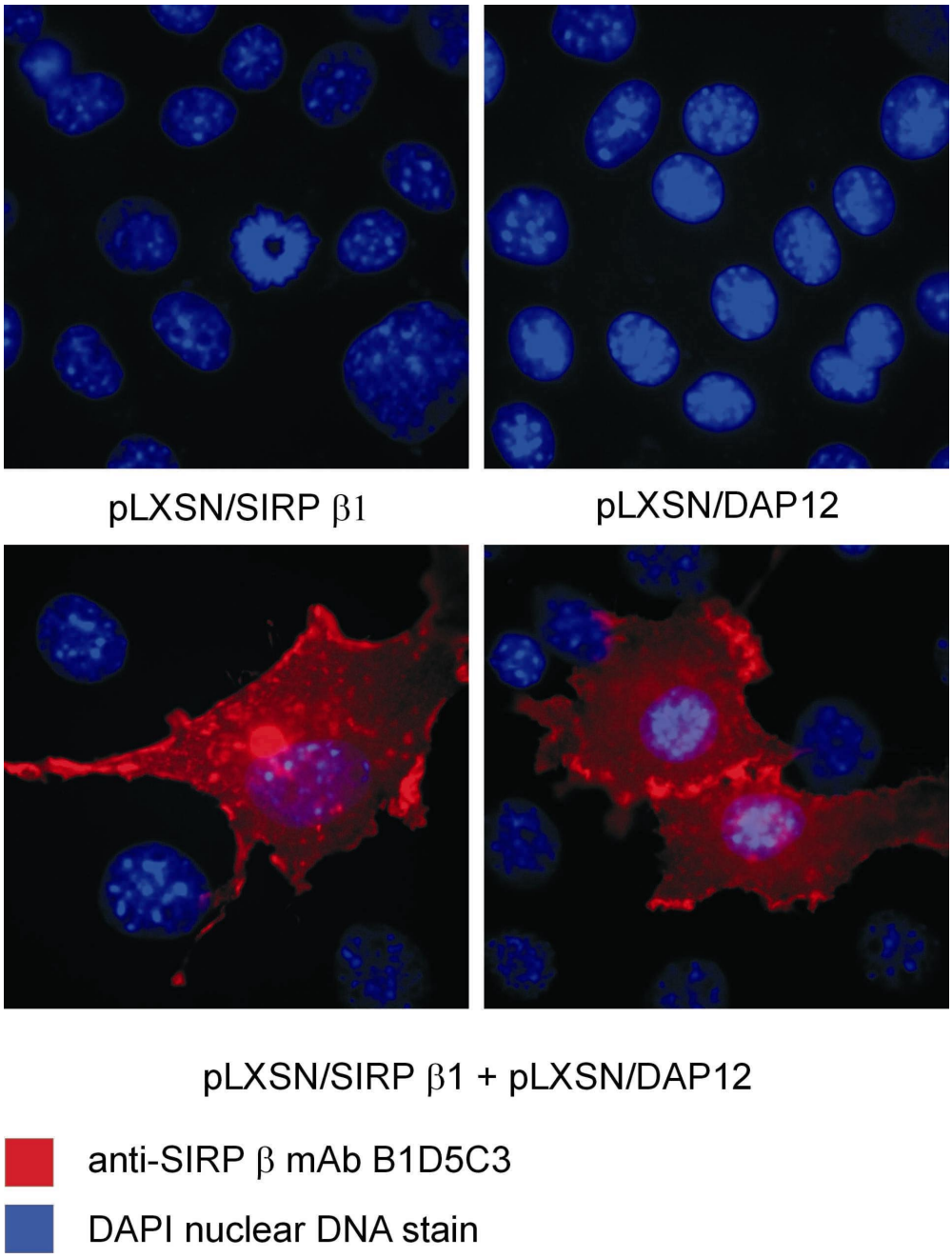


Figure 26 Stability of SIRP β1 relies upon the coexpression of DAP12. NIH3T3 cells transfected with different pLXSN constructs (as indicated in the Figure) incubated with mAb B1D5C3 to detect overexpressed SIRP β1. Antibody was detected by incubation with CY3 (red) coupled goat-anti-mouse antibody and visualised by immunofluorescence microscopy (λ_{ex}=552 nm, λ_{em}=570 nm). Nuclear DNA was stained by DAPI (blue) (DAPI: λ_{ex}=358 nm, λ_{em}=461nm).

Use of the mAb B1D5C3 in immunofluorescence confirms that at lower expression levels, SIRP β1 is undetectable in the cell. When DAP12 is also overexpressed,

SIRP β1 expression is stabilised, fitting with the experiment shown in Figure 25, and localised to the plasma membrane. The subsequent experiment shown in Figure 27 suggests that SIRP β may exit the cell when not tethered to the membrane through its association with DAP12.

Figure 26 confirms the evidence presented in the previous figures with immunofluorescence. SIRP β1 can only be visualised when both SIRP β1 and DAP12 are expressed together in the cell. The localisation of SIRP β1 is principally at the membrane, suggesting that SIRP β1 moves from the ER to the membrane with DAP12 (see Figure 23). Additionally, it appears that the signal is stronger at points of cell-cell contact (bottom-left picture) that may point to a role in intercellular communication.

The intracellular domains of single-pass transmembrane proteins often serve as anchors that prevent their secretion into the extracellular milieu. SIRP β1 has no intracellular domain, but travels to the plasma membrane with DAP12. If SIRP β progressed to the cell membrane in the absence of DAP12, it may therefore be secreted. Alternatively, SIRP β may be restricted in the ER. Both situations would produce the result seen in Figure 22.

To determine whether the fate of SIRP β in the absence of DAP12 lies in the ER or in the extracellular milieu, SIRP β1 was overexpressed with or without DAP12, and cell lysates as well as cell culture supernatants were tested for the presence of SIRP β.

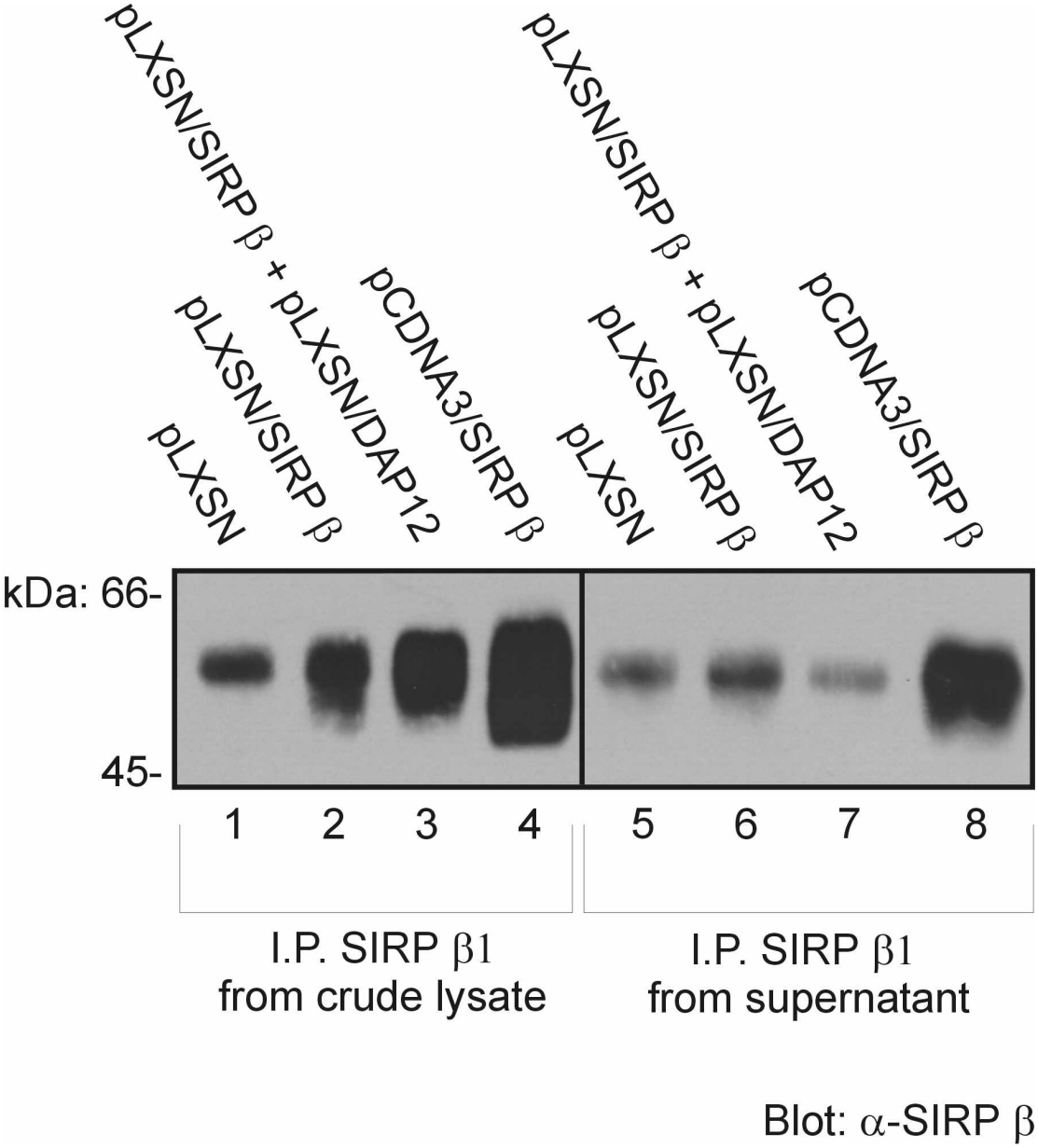


Figure 27 Secretion of overexpressed SIRP β is impeded by DAP12 overexpression. HEK293 cells transfected with pLXSN constructs overexpressing SIRP β1 (lanes 2-3, 6-7) and DAP12 (lanes 3,7) or with pCDNA3/SIRP α1 (lanes 4 and 8). SIRP α1 was immunoprecipitated from balanced cell lysates (lanes 1-4) or equal volumes of cell culture medium (lanes 5-8). Proteins were separated on 10% SDS PAGE and detected in Western blot using anti-SIRP β antibodies.

When SIRP β1 is overexpressed under the control of a strong CMV promoter (as in Figure 22), abundant quantities of SIRP β1 are found in the supernatant fraction (lane 8). Even at LTR-driven levels, however, SIRP β1 is detectable in the supernatant (lane 6).

SIRP β1 expression in cell lysates is increased or stabilised when DAP12 is co-expressed while it is reduced in the supernatant (compare lanes 2-3 and 6-7). The data suggests therefore that overexpressed SIRP β1 is passively secreted, and that coexpression of DAP12 restricts SIRP β1 secretion by anchoring it in the cell membrane.

5.5 Caveolar Localisation of SIRP proteins

SIRP β1 and DAP12 were both detected at the cell membrane when both expressed, and there was an inhibitory influence of SIRP α upon the SIRP β/DAP12 complex. It was therefore theorised that both subtypes may operate in close proximity in the cell.

SIRP α are thought by some to be scaffold proteins[23], and are involved in the signalling of many proteins (e.g. RTKs, src-family kinases) that are found in organised raft structures, or caveolae. Caveolae are subcellular structures in the plasma membrane thought to be responsible for the spatial organisation of multiprotein complexes in signal transduction[71]. It was decided to test for the presence of SIRP family members in caveolae.

Two methods were employed to isolate caveolar fractions from the rest of the cell membrane. In both cases, caveolae-containing fractions were identified using an antibody raised against the caveolar marker protein, Caveolin1.

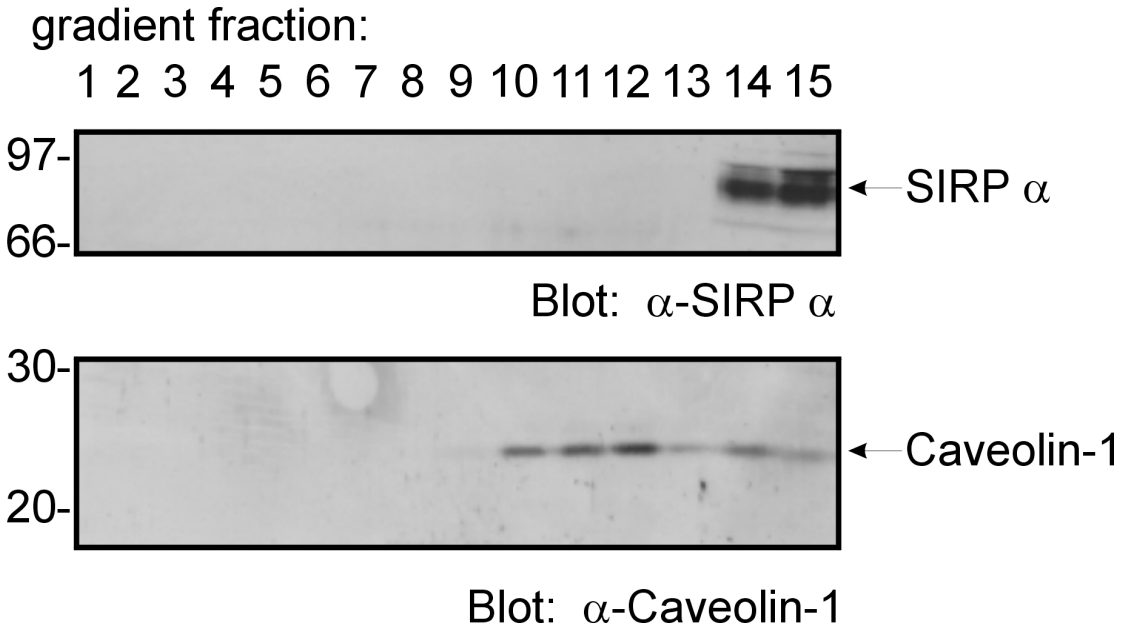


Figure 28 Separation of Triton-X-100 insoluble fractions on sucrose density gradient. Sub-confluent MCF-10A cells growing in medium with 10% serum were lysed in buffer Triton-X100 and the lysates were separated by sucrose density gradient ultracentrifugation. Fractions of 0.5 ml were taken from the top of the tube (fraction 1) to the bottom (fraction 15). Fractions were separated on 8% - 16% SDS PAGE and proteins were detected in Western blot with polyclonal antisera recognising SIRP α (top panel) and Caveolin1 (bottom panel).

Figure 28 shows that SIRP α is not enriched in caveolae-containing fractions (fractions 10-12) prepared by lysing cells in Triton-x-100 and separating the buoyant insoluble fraction from the pellet by density gradient ultra centrifugation. The fractions containing caveolae are identifiable by the intensified Caveolin1 signal in the Western blot (lower panel, lanes 10-12). The total extent of the detectable SIRP α signal resides, however, in the pellet (fractions 14 and 15) as is clear in the upper panel. However, there is evidence that this method of caveolus isolation has a disruptive effect on its structural integrity because many more proteins can be found in the caveolar fractions prepared using detergent-free methods[71].

To find out whether or not SIRP family proteins can be found in the Triton X-100 – soluble component of the caveolus, a detergent-free method was used.

This method involved homogenising cells and separating the plasma membrane fractions from the remainder of the cellular material on a Percoll® gradient. The plasma membrane is then sonicated and the buoyant, Caveolin1-containing

fractions are separated from the remainder of the plasma membranes on a second Optiprep® gradient.

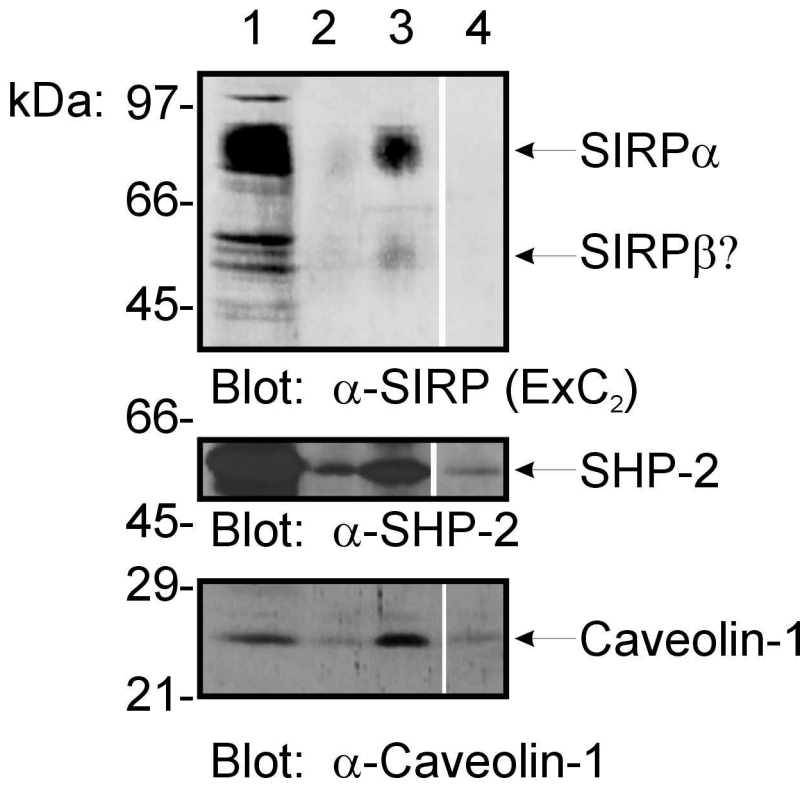


Figure 29 SIRP proteins are present in caveolae-containing fractions. Representative fractions are depicted from detergent-free isolation of Caveolin-containing fraction of MCF10A cells growing in medium with 10% serum. Fractions were separated with 8% to 16% SDS PAGE. Loading of lanes was balanced for representative initial cellular content. Lane 1, crude cell lysates prepared by treatment of cells with 1% SDS lysis buffer. Lane 2, plasma membranes isolated by Percoll gradient separation. Lane 3, buoyant fraction following sonication of membranes and separation by Optiprep gradient. Lane 4: remainder of plasma membrane fraction after removal of buoyant fraction.

Caveolae-containing fractions produced by this method contain SIRP α molecules because SIRP α proteins are clearly enriched with Caveolin1 in the same fraction (Figure 29). Antibodies against SIRP α also detected a diffuse signal between ~45 and ~66 kDa, which is the size of SIRP β. This suggests that SIRP proteins are indeed part of the contents of the raft, or caveolus.

5.6 Biological Effects of the SIRP β/DAP12 complex in NIH3T3 cells

5.6.1 NIH3T3 derived cell lines overexpressing SIRP β/DAP12

Since coexpression of SIRP β1 and DAP12 are needed to establish a stable complex at the cell membrane, study of biological potential is best achieved by comparing cell lines possessing both SIRP β and DAP12 with mock transfected cell lines. To this end, NIH3T3 cells were infected with either the empty pIRES or pIRES/SIRP β1+DAP12. Stable G418-resistant monoclonal cell lines were screened for expression of both SIRP β1 and DAP12 and three clones with detectable expression of both proteins were chosen and compiled into one oligoclonal cell line (SD469) with the advantages of the enhanced stability of expression of the gene of interest associated with a monoclonal cell line and the reduced likelihood of artefacts associated with a polyclonal cell line (Figure 30)

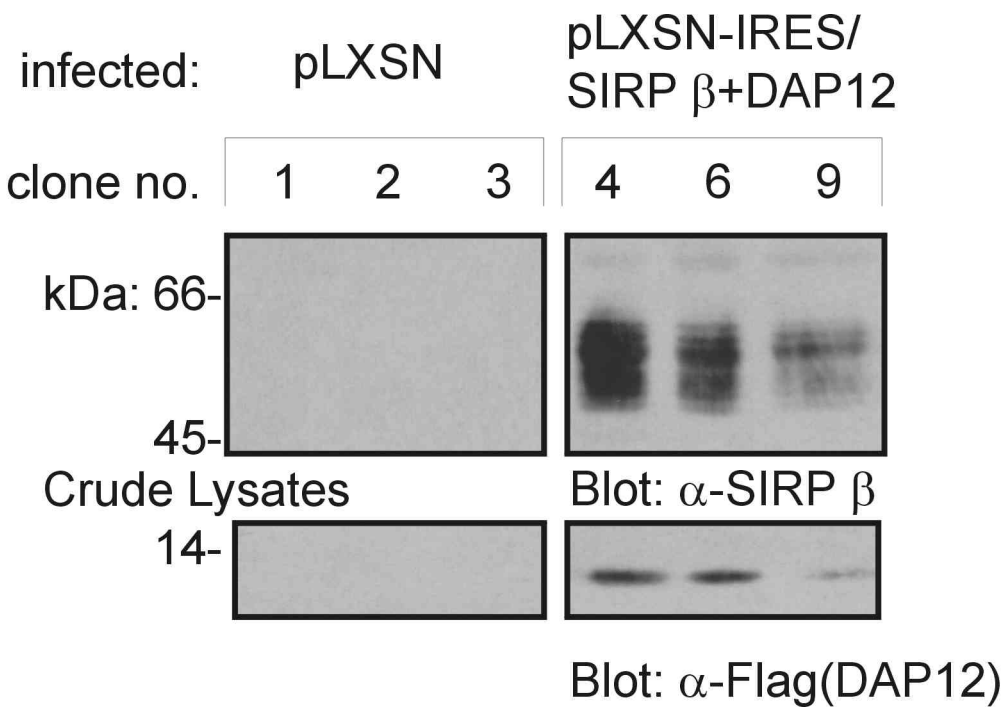


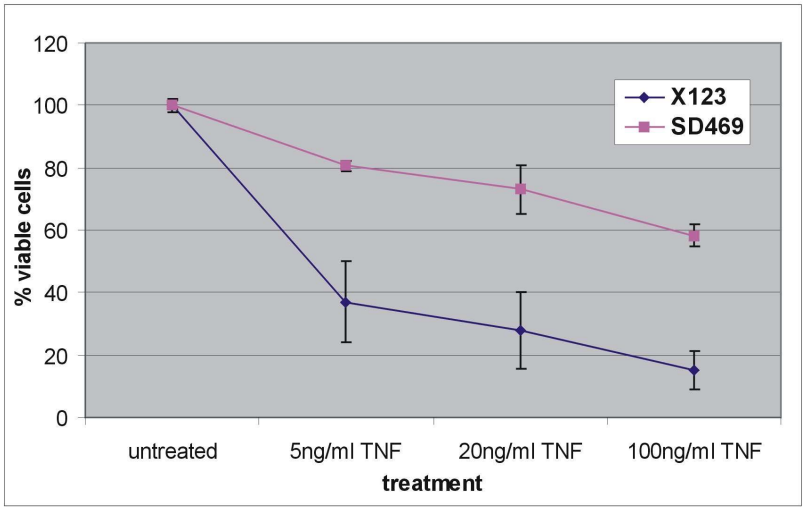
Figure 30 The six clones used to produce the two NIH3T3-derived oligoclonal cell lines X123 and SD469. NIH3T3 cells were infected with pLXSN (left block) or pIRES/SIRP β1+DAP12 (right block). Cells were selected in G418 for 14 days, then dispersed sparsely over fresh plates. 10 clones were picked and grown in isolation. Each clone was tested by Western blotting with antibodies against SIRP β (upper pictures) or DAP12 (lower panel). The oligoclonal cell line X123 is a compilation of the three monoclonal pLXSN-infected clones 1,2, and 3. The SIRP β1/DAP12-overexpressing cell line SD469 is a compilation of the monoclonal pIRES/SIRP β1+DAP12-infected clones 4,6,and 9 (see above).

Two phenotypic observations could be distinguished in the NIH3T3 cells overexpressing SIRP β and DAP12 (SD469) from the control cell line (X123): a resistance to induced-cell death and enhanced rate of acid production. These observations are described in the following sections, where for the purposes of simplicity are sometimes collectively referred to as the “SD469 phenotype”.

5.6.2 Resistance to cell death induced by different stimuli

5.6.2.1 TNFα stimulation

A



B

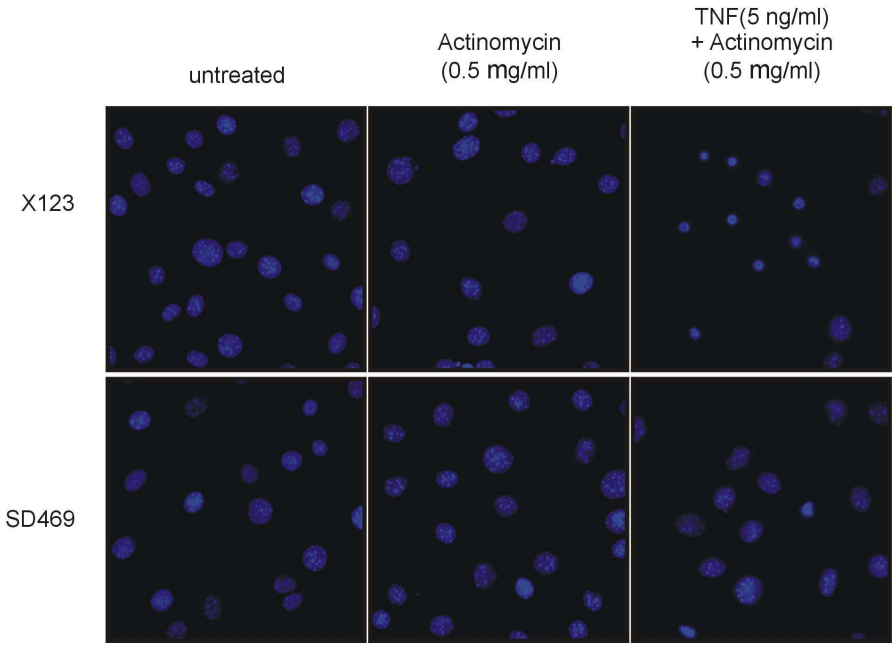


Figure 31 Resistance of an NIH3T3-derived cell line overexpressing both SIRP β1/DAP12 (SD469) to TNFα-induced cell death. (A) Comparison is with that of a control cell line (X123). $2.5x10^3$ cells were seeded in 96-well plates and grown for 48 hours. Cells were starved for 18 hours in medium containing 0.5% serum before stimulation with indicated quantities of TNF α in the presence of actinomycin D. After a further 18 hours, viable cells were measured by MTT assay and are presented as a percentage of the untreated value. (B) Samples treated in the same manner were seeded on cover slips, fixed, and stained with DAPI. The nuclei were examined by fluorescence microscopy (lower pictures).

Figure 31 shows that SD469 are resistant to TNFα-induced cell death when compared with a mock-infected cell line (X123). Experiments with other established apoptosis-inducing conditions were performed to determine the specificity of this effect for TNFα. The lower part of the Figure illustrates through the examination of the nuclei after DNA staining with DAPI that treatment with TNFα leads to the nuclear condensation (which is characteristic of the onset of apoptosis) to a far greater extent in control cells than in cells overexpressing SIRP β/DAP12.

5.6.2.2 Depletion of serum

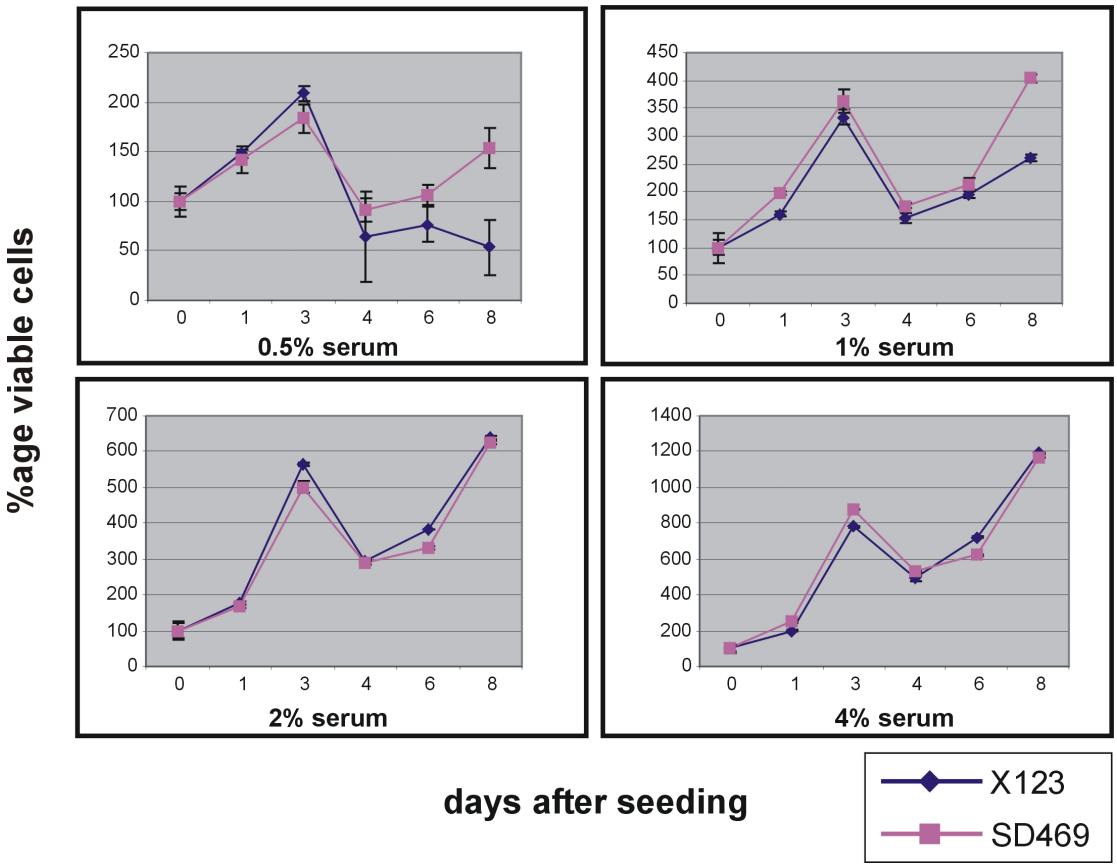


Figure 32 Culture of control (X123) or SIRP β/DAP12 overexpressing NIH3T3 cells in DMEM medium containing different serum concentrations. X123 or SD469 cells were grown for 8 days in DMEM medium with 4 different concentrations of serum. After each day, the number of viable cells was measured by MTT assay and represented as a percentage of the initial cell number.

When cells are grown at serum concentrations of 0.5% or 1%, there is a divergence in the viability of the two different cell lines after 4 days (Figure 32). The control cells (X123) appear to lose viability, while the cells overexpressing SIRP β1/DAP12 are able to survive for longer. This effect may be accredited to an inhibition of apoptosis concurrent with the experiment result shown in Figure 31. While this effect is visible at lower serum concentrations, it is overcome at higher concentrations, suggesting that what is being measured is the response to a stress stimulus upon the cells and not representative of the overall fitness of the cells.

5.6.2.3 Ultraviolet (UV) irradiation

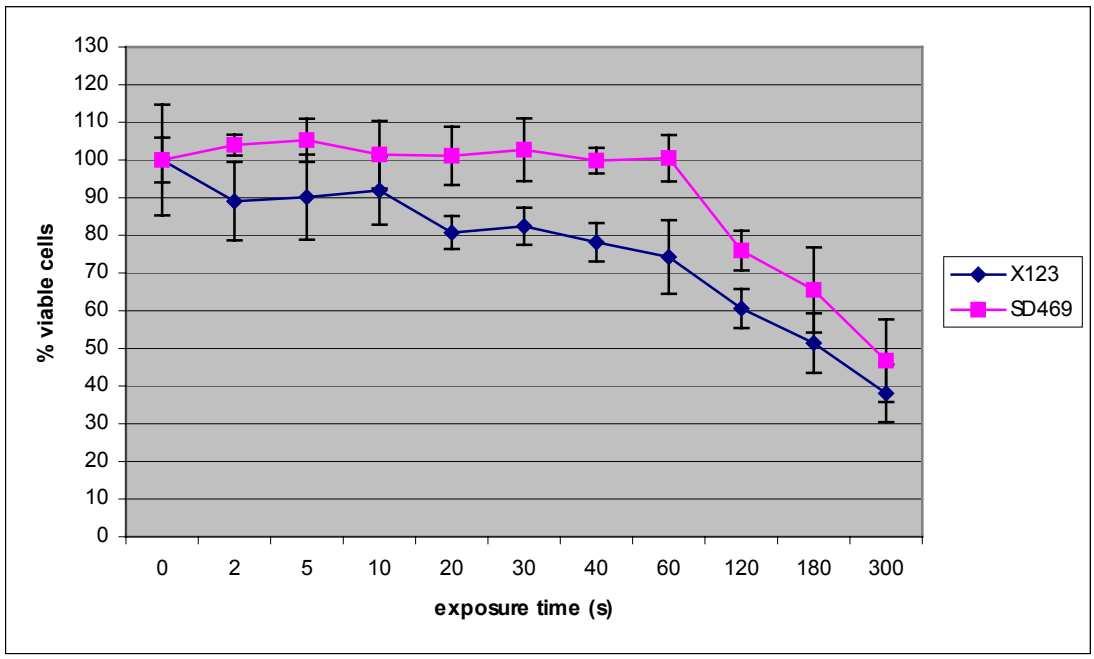


Figure 33 Resistance of cells overexpressing SIRP β1/DAP12 to UV-induced cell death with respect to control. Cells were seeded in 96-well dishes (2000 per well) and exposed to the UV light of a cell culture laminar flow for increasing periods of time. The cells were left overnight and an MTT assay was performed the following morning to assess cell viability, which was presented as a percentage of the viability of untreated cells.

Although a weaker effect is produced by UV irradiation, there is nevertheless a trend to represent a resistance to UV-induced cell death in cells overexpressing SIRP β1/DAP12.

5.6.3 Influence of inhibitors on resistance to cell death

Since treatment by TNFα was the most convenient and effective way to induce cell death in this system, all subsequent experiments were performed using TNFα treatment. The experiments were repeated in the presence of inhibitors of PI-3K and src-family kinases to try to implicate them in the mechanism leading to this phenotype.

5.6.3.1 PI-3K inhibitor Wortmannin

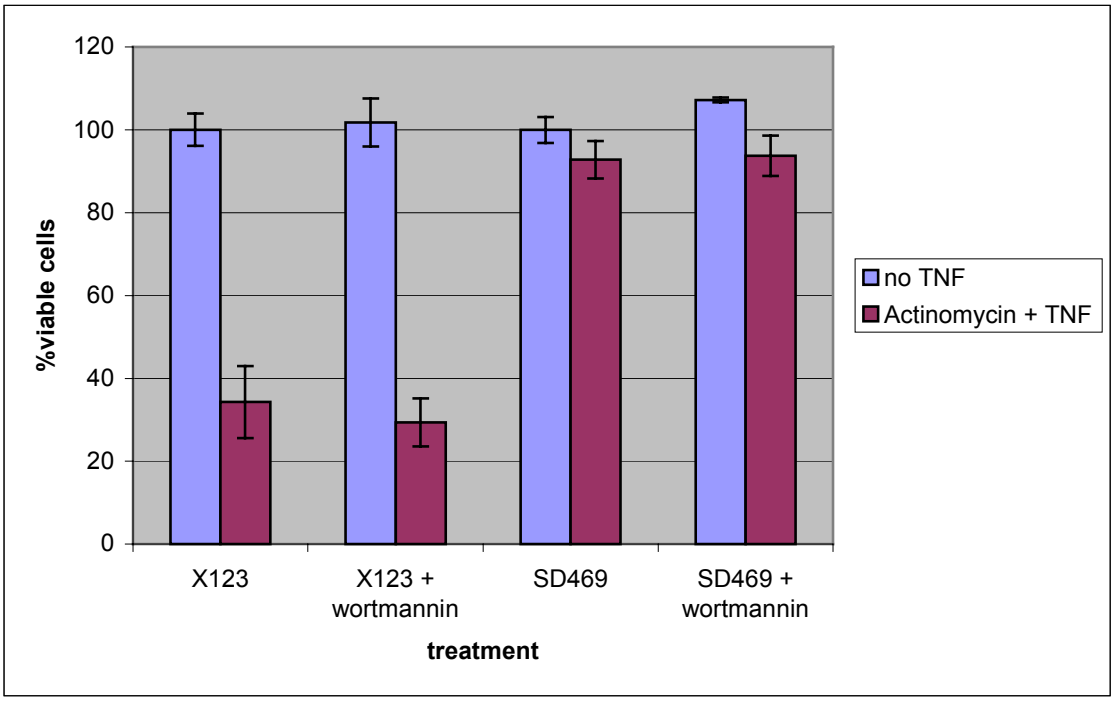


Figure 34 Resistance of SIRP β1/DAP12 overexpressing cells to TNFα-induced cell death is unaffected by Wortmannin treatment. Cells were grown in DMEM containing 10% serum, starved in DMEM containing 0.5% serum then stimulated overnight in medium containing 20 ng/ml TNFα and 500 ng/ml Actinomycin D. Wortmannin was included in the medium to an end concentration of 5μM where indicated. Viable cell number was measured using the MTT assay and assessed as a percentage of that of untreated cells.

Since there is a biochemical link between the activity of Syk and phosphoinositide 3-kinase (PI-3K), and that PI-3K activates protein kinase B, or Akt, the activation of which is, in turn, able to inhibit apoptosis through the Bcl/Bax family of proteins, one may hypothesise the involvement of PI-3K in the resistance to induced cell death. Incubation with the classical inhibitor of PI-3K, however, is ineffective in abrogating this resistance to apoptosis, (Figure 34) suggesting that SIRP β1/DAP12 does not produce these effects using this pathway.

5.6.3.2 Src-family kinase inhibitor PP1

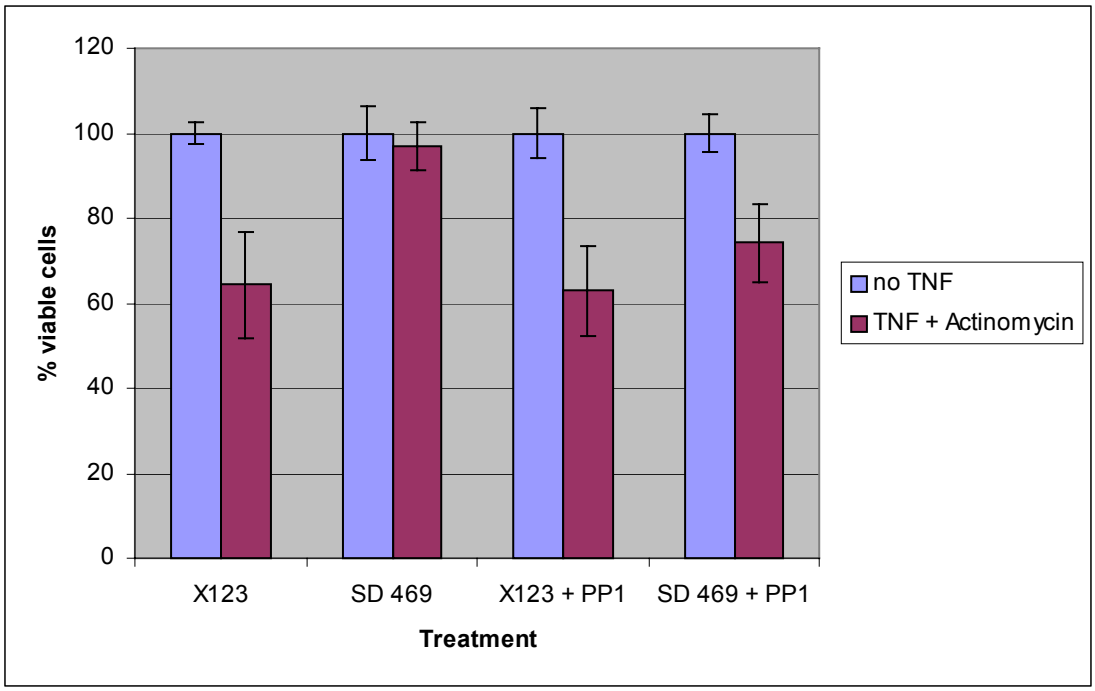


Figure 35 Resistance of cells overexpressing SIRP β1/DAP12 to TNFα-induced cell death is inhibited by high concentrations of PP1. Cells were grown in DMEM containing 10% serum, starved in DMEM containing 0.5% serum then stimulated overnight in medium containing 20 ng/ml TNFα and 500 ng/ml Actinomycin D. PP1 was included in the medium to an end concentration of 80 μM where indicated. Viable cell number was measured using the MTT assay and assessed as a percentage of that of untreated cells.

Although Wortmannin was unable to suppress the resistance of SIRP β1/DAP12 overexpressing cells to TNFα-induced cell death, treatment of cells with the src-family kinase inhibitor was successful, and the inhibition of the phenotype increased in a dose-dependent fashion, although the effects began at PP1 concentrations higher (8-fold) than necessary to inhibit src-family kinases, the established concentration being 10 μM.

5.6.4 Role of Syk in resistance to cell death

Following the identification of Syk as a member of the SIRP β/DAP12 complex it was necessary to determine the role, if any, that Syk may be playing in cell death resistance by overexpressing a kinase inactive form of Syk.

To this end, the ORF of Syk was cloned from cDNA by PCR, and a K402R substitution was produced by site-directed mutagenesis that removes the ATP-binding site of the protein and hence abolishes the kinase activity. The plasmid clones following the *in vitro* mutagenesis were overexpressed in HEK293 cells and tested for the kinase activity of the expressed protein.

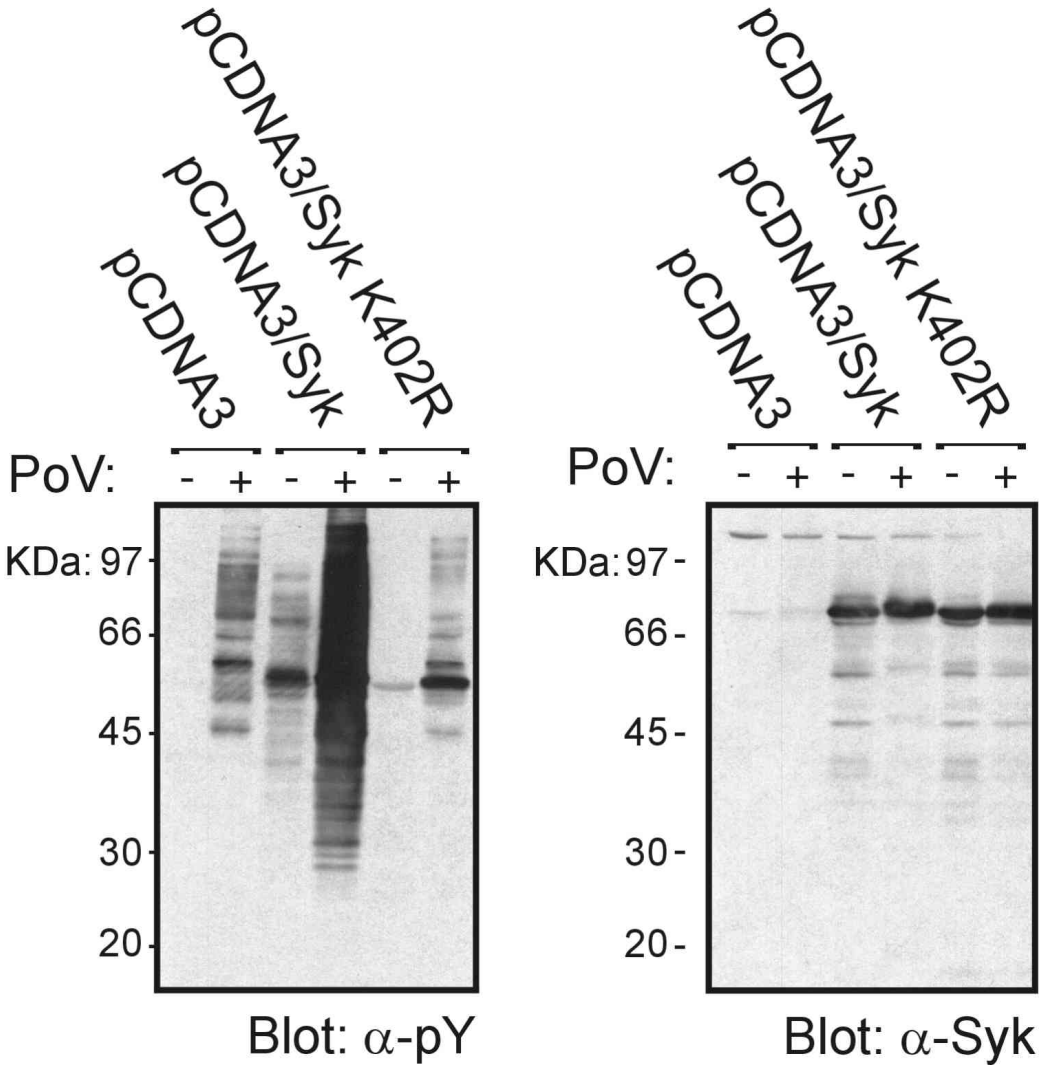


Figure 36 Construction of a kinase-inactive Syk. Syk was amplified by PCR cloning from cDNA prepared from HEK293 cells. The coding region was subcloned into pCDNA3 and the lysine at position 402 was mutated to an arginine to produce pCDNA3/Syk K402R by site-directed *in vitro* mutagenesis. HEK293 cells were transiently transfected with pCDNA3, pCDNA3/Syk or pCDNA3/Syk K402R as indicated. Cells were stimulated with 1mM sodium pervanadate (PoV) for 10 minutes before lysis. Proteins were separated on 10% SDS PAGE and detected in Western blot with anti Syk antiserum (left picture) or anti-phosphotyrosine antibodies.

Overexpression of Syk in HEK293 cells leads to the increased tyrosine phosphorylation of a large number of proteins, ranging mainly from 87 kDa to 45 kDa (Figure 36, left picture). Inhibition of tyrosine phosphatases by sodium pervanadate treatment enhances this effect greatly. In contrast, there is very little tyrosine phosphorylation produced latently by Syk K402R and pervanadate treatment produces a range of tyrosine phosphorylated proteins that is reduced

with comparison to pCDNA3-transfected cells. It is concluded from this evidence that Syk K402R is kinase-inactive.

To test the influence of Syk on the resistance of SIRP β1/DAP12 overexpressing cells to TNFα-induced cell death, polyclonal cell lines were produced by transfecting either X123 or SD469 cells with pCEP4, pCEP4/Syk or pCEP4/Syk K402R. Cells were selected with hygromycin, and tested for their reaction to TNFα stimulation (Figure 37).

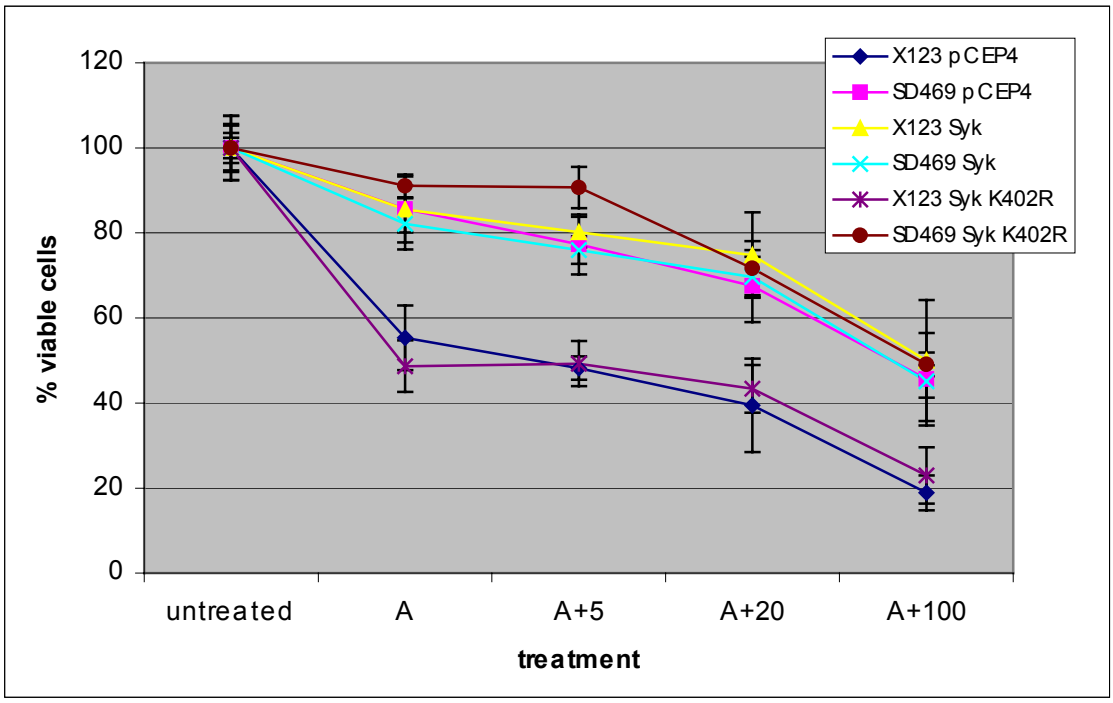


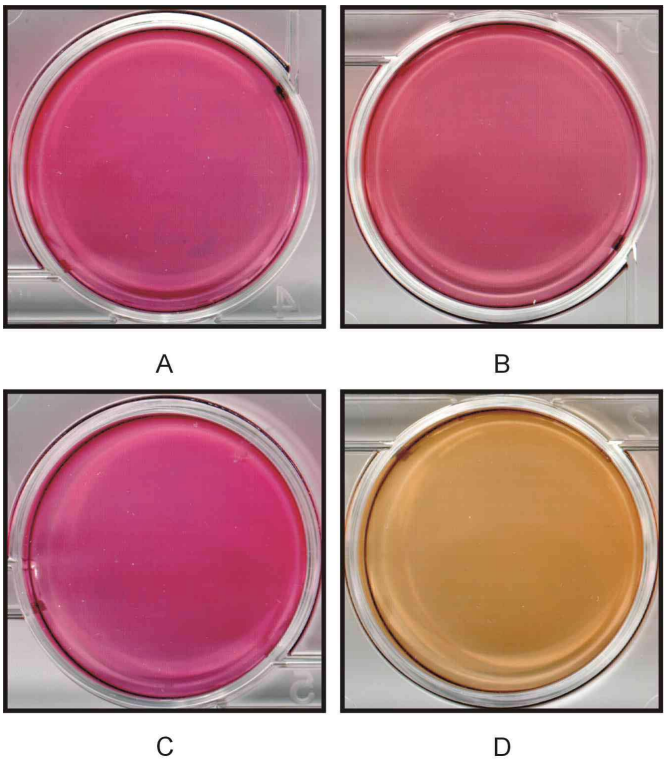
Figure 37 Overexpression of wt Syk in NIH3T3 control cells is sufficient but not necessary to reproduce the SD469 phenotype. The figure represents the response of the six stable cell lines (legend, top-right) to treatment with different concentrations of TNFα. The different treatments were: "A"(500 ng/ml actinomycin D), A+5 (actinomycin D+ 5ng/ml TNFα), "A+20" (actinomycin D + 20 ng TNFα) and "A+100" (actinomycin D + 100 ng/ml TNFα). Cells were treated overnight and cell viability was quantified using the MTT assay and represented as a percentage of that of the untreated cells.

Figure 37 shows two distinct trends produced from the cytotoxicity assay. The resistance of SIRP β1/DAP12 overexpressing cells to TNFα-induced cell death is reproduced in the mock-transfected X123 and SD469 cells. Secondly, kinase-inactive Syk overexpression does not alter the phenotype of the cells in either X123- or SD469-derived cell lines. However, overexpression of Syk in X123 cells

produces a similar resistance to TNFα as SD469 cells. The resistance produced by SD469 cells is not, however, enhanced by overexpression of Syk. This suggests that overexpression of Syk is sufficient but not essential for resistance to TNFα-induced cell death, and therefore that the effect produced by SIRP β1/DAP12 overexpression may operate via a different pathway.

5.6.5 Enhanced glucose metabolism

While the SD469 and X123 cells were in culture, a difference in colour of the cell culture medium was observed. Measuring the pH of the medium disclosed a clear difference after two days of culture (Figure 38).



A: X123 in low glucose
B: X123 in high glucose
C: SD469 in low glucose
D: SD469 in high glucose

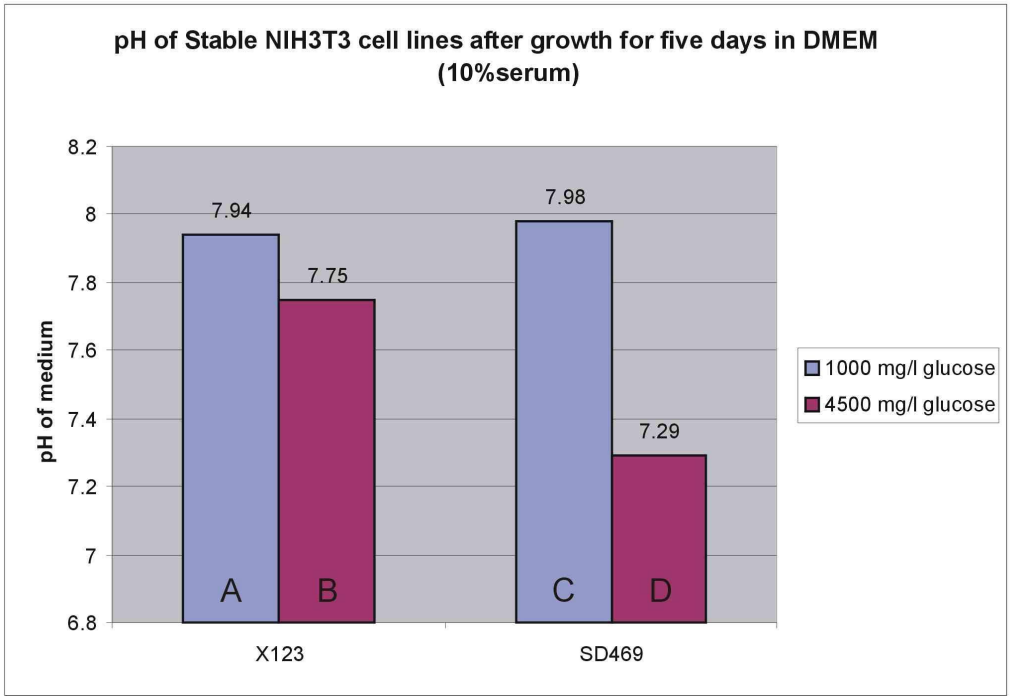


Figure 38 Enhanced acid production in NIH3T3 overexpressing SIRP β/DAP12 with comparison to control. Confluent X123 (A and B) or SD469 (C and D) cells were cultured for 48 hours in DMEM medium containing either 1000 mg/l glucose (A and C) or 4500 mg/l (B and D) glucose. The pH of the medium was measured with a Microprocessor pH/Ion meter (WTW pMX 3000). The colour of the cell culture medium was photographed for illustration.

The drop in medium pH over two days of culturing SD469 was significantly greater than in X123 cells that was inhibited when the cells were incubated in medium with a lower concentration of glucose. This enhanced rate of production of acid into the cell culture medium therefore suggests a higher rate of glucose metabolism that would suggest enhanced rates of glycolysis.

To establish whether enhanced acid production maybe reflected a higher rate of glucose usage, glucose uptake was measured. Cells were additionally stimulated with PDGF, which has been established to induce glucose uptake in NIH3T3 cells[72].

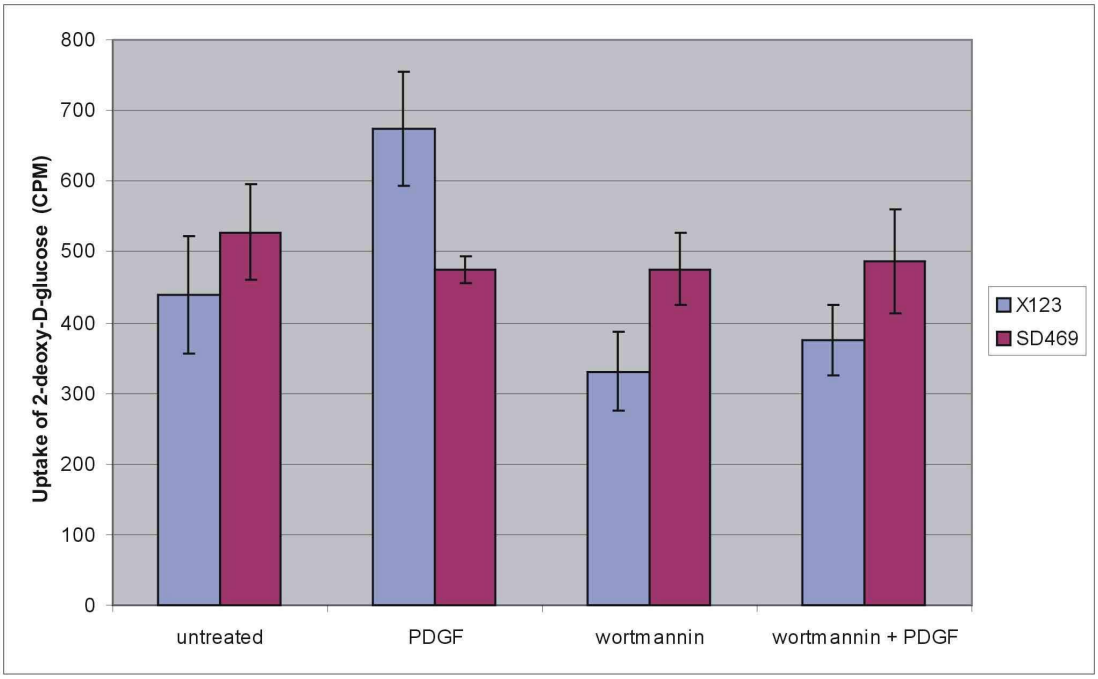


Figure 39 Enhanced glucose uptake in cells overexpressing SIRP β/DAP12. X123 or SD469 cells were stimulated with or without PDGF B/B in the presence or absence of 5 μM Wortmannin for 1 hour (50ng/ml final concentration) after which ^{3}H-2-deoxy-D-glucose was added to a final concentration of 5μCi/ml. After 5 minutes cells were washed and lysed and incorporation of ^{3}H was measured.

The experiment above suggests that enhanced acid production occurs concomitantly with a slightly enhanced rate of glucose uptake. This was insensitive to Wortmannin, suggesting that PI-3K was not involved in this effect. Additionally, in contrast to enhanced glucose uptake following treatment of X123 with PDGF, SD469 appear to be insensitive to PDGF stimulation.

6 Discussion

6.1 Association of SIRP α extracellular domain with CD47 and other molecules

The role of the extracellular domain was addressed by the use of eukaryotically produced SIRP extracellular domain GST fusion proteins in adhesion assays.

The results revealed little difference between the adhesion properties of SIRP α1 and SIRP α2. These two SIRP α family members are 98.5% identical in primary protein sequence, only 13 amino acids are different, and of these, only 7 produce a change in the charged nature of the amino acid side chain. All of these substitutions are in the extracellular domain of the protein, however, the binding characteristics of SIRP α1 and SIRP α2 means that these differences are not sufficient to cause the binding specificity to differ between the two SIRP α family members.

SIRP β1 also shares a high degree of identity in primary protein sequence to SIRP α1 and SIRP α2 though not quite as high as that between SIRP α1 and SIRP α2. With the exception of the secretory signal peptides, which are totally different and are probably cleaved in the mature protein, SIRP β1 is 90% identical and 94% similar to SIRP α1 in the extracellular domain. These differences are however, enough to bring about differences in the binding specificity of SIRP β1, as seen in the cell adhesion experiments performed here. The confirmation that SIRP α1 and SIRP α2(not shown) but not SIRP β1 bind to CD47 also illustrates this. Therefore, small differences that are evident amongst all SIRP family members can in some cases bring about altered ligand specificity. Ligand affinity could also be modified by different patterns of glycosylation. Of the amino acid differences between SIRP β1 and SIRP α1, at least one of them either creates or destroy a potential glycosylation site Both primary sequences contain the putative N-glycosylation motif NXS/T ^{73}5 times at the same position, however, there is an extra N-glycosylation site at amino acid position 105 of the SIRP β1 sequence that is not present in SIRP α1.

The difference between the adhesion characteristics of GST-fused and IgG-fused extracellular domains of the same family member must be explained. The internal control, GST, produced little or no cell adhesion. Additionally, neither SIRP β1exGST nor SIRP β1exIgG supported cell adhesion. This shows that the cells are binding to the SIRP extracellular domains and not to either of the tags. Therefore, something else produces the difference in some cell lines between SIRP αexGST and SIRP αexIgG.

The method of purification of the GST fusion proteins is different to that of the IgG fusion proteins and may provide the answer. After binding to the affinity column, the GST fusion protein is eluted by soluble glutathione, which competes with the glutathione bound to the column for active sites on the GST tag. The GST fusion protein is therefore removed by a method that does not involve denaturation of the protein. However, the IgG fusion proteins are eluted with a low pH buffer, which disturbs charged interactions between the side chains of the bound protein and its ligand. The buffer is subsequently neutralised to return the protein to its original state. The conformation of a protein can also be stabilised by intramolecular interactions between charged amino acids. Disrupting these interactions might force an irreversible change in the conformation of the protein - a partial denaturation.

Despite this putative denaturation, in most cases both SIRP α1exGST and SIRP α1exIgG support cell adhesion equally well. However, when certain cell lines are used, adhesion is abolished. If SIRP α had only one ligand, mild denaturation would produce an overall reduction in affinity for the ligand and the adhesion pattern of the IgG fusion proteins would reflect those of the GST fusions but with weaker binding. As we have seen, this is not the case here. The actual situation would be explainable if there were two different ligands (L_s and L_i) binding to SIRP α molecules at different sites (B_s and B_i, respectively). If B_s was sensitive to treatment at low pH and unable to renature, but B_i was insensitive to the treatment, the IgG fusions would only be able to bind to L_i while the GST fusions would still bind to L_s and L_i. The difference in adhesion pattern would therefore be representative of the relative expression of L_s.

The adhesion of OV10/x cells not expressing CD47 to SIRP α1exGST can be explained by this theory. CD47 would be L_i, the ligand for the insensitive binding site, because both SIRP α1exGST and SIRP α1exIgG support adhesion of OV10/ CD47. But cells that adhere to SIRP α1exGST despite the absence of CD47 would be expressing L_s, the ligand for the sensitive binding site(B_s). According to the theory OV10 do not adhere to SIRP α1exIgG because B_s was destroyed during elution.

The likelihood that SIRP α has more than one ligand is supported by other characteristics of SIRPs: The existence of a family of proteins that differ only very slightly in their primary sequence suggests a protein-ligand relationship that is subtly specific, and may involve contact with another family with a similar degree of polymorphism. The existence of a potential splice variant of SIRP α that lacks the second and third c-type Ig-like loops provides additional evidence for more than one ligand for SIRP α. Assuming that this region is responsible for an inter- and not intramolecular association, this splicing is likely to remove at least one binding site for another molecule.

CD47, also known as 'integrin associated protein (IAP)', was recently identified by others and ourselves[21,74] as a ligand for SIRP α. It is a five transmembrane-spanning glycoprotein present in a variety of cells of neural and haematopoietic lineage and other tissues and is co-expressed with SIRP α in retinal cells[75]. The pre-mRNA is alternatively spliced leading to protein isoforms with intracellular deletions of different sizes[47,76-78]. CD47 has been implicated in cell adhesion and transepithelial cell migration. Apart from SIRP α, it also associates with the $\alpha_v\beta_3$ integrin (vitronectin receptor) [79], 'proteins linking IAP with the cytoskeleton' (PLICs) and thrombospondins[47]. The interaction between SIRP α and CD47 has now been implicated in a number of processes.

Amongst other haematopoietic cells, CD47 is also expressed in red blood cells. Oldenborg et al.[80]used erythrocytes from a CD47 knockout mouse to show that normal macrophages, which express SIRP α, kill red blood cells that have no CD47 expression. The authors speculate that CD47 is a 'marker of self' that the immune system uses to distinguish self from non-self. However, this is likely to be

understating the role of the CD47/SIRP α interaction because it is probably involved in other systems. First of all, both CD47 and SIRP α are expressed outside the haematopoietic system, notably the brain. A bi-directional signalling relationship involving SIRP α and CD47 has been discovered in the retina[75], for example.

Other models involving the interaction between SIRP α and CD47 have been studied within the haematopoietic system. Macrophages express both CD47 and SIRP α, and one study proposed that the interaction between them facilitated macrophage fusion and multinucleation[81,82]. There are also hints that the same interaction may facilitate transendothelial cell migration [21].

Although the interaction has not been precisely characterised, the binding site for CD47 has been mapped to the N-terminal Ig-V like domain of SIRP α[83]**.** CD47 does not belong to a family of closely related molecules like SIRP α so its binding is probably not influenced by the subtle variation between SIRP α family members. It has to be assumed, of course, that the variation among SIRPs has functional significance, and is not the result of passive degradation following a recent gene duplication event. With this in mind, it is interesting to speculate upon other possible ligands for different SIRP α molecules. Different SIRP α family members may have altered specificity for other transmembrane spanning molecules that belong to a family with similar variety in the extracellular domain. A similar hypothesis has already been speculated about the KIR family and different species of MHC class I ligands[84]. SIRP α resides constitutively in a complex with the CSF-1 receptor in macrophages[22] and SIRP α may bind to the PDGFR itself[85] so certain RTKs of the PDGFR family, which are also Ig-superfamily members, could therefore be candidate ligands for SIRP α molecules.

Adhesion assay experiments suggest that CD47 is not a ligand for SIRP β, because SIRP β1exGST does not support adhesion of OV10/CD47 cells. This may be true, but it has to be assumed when one draws this conclusion that the SIRP β1exGST and SIRP β1exIgG really resemble the native SIRP β in the extracellular domain. SIRP β associates to DAP12, which is a dimeric molecule, and therefore has two binding sites for a SIRP β. A functional SIRP β/DAP12

complex may therefore be tetrameric, containing 2 SIRP β molecules that are close together and spatially aligned. If this is necessary for effective ligand binding, the situation is very probably not reflected by immobilised GST fusion proteins.

While most tested cell types were able to adhere to SIRP α1exGST and SIRP α1exIgG, only one cell type showed any adhesion to SIRP β1exGST, and no cells could adhere to SIRP β1exIgG. This could either mean that SIRP β has no ligand, or that the affinity or avidity of SIRP β for its putative ligand is below the detection threshold of adhesion assay. Northern and Western blotting data [70] suggest that SIRP β is a weakly expressed protein in all cell types studied. If SIRP β contacts a ligand unique for itself, one would expect that the ligand might be expressed at a similar level. In this case, the affinity of a putative ligand for SIRP β1exGST may be below the detection threshold of the adhesion assay, even if the concentration of SIRP β1ex were high. If this were the case, expression cloning in the manner by which CD47 was isolated as a ligand for SIRP α [74] would be a realistic option because the ligand would be overexpressed and the avidity would be enhanced.

Although SIRP α contact with its ligand is often strong enough to sustain cell adhesion in an adhesion assay, this contact probably does not occur in isolation *in vivo*, but together with a number of different coreceptors each contacting their own ligands, leading to a much stronger cytostructural affinity. Indeed, SIRP α-ligand interactions may not play structural roles, but signalling roles on the cell to elicit a program of cell adhesion. In this context, it is easy to bring in the possible role of the SIRPs as 'cellular signposts' on the surface of the cell, indicating perhaps to a migrating lymphocyte or extending axon that it is in the right or wrong place, and aiding to direct it accordingly.

It is tempting in this context to consider SIRP β as a possible chemorepellant because of two observations. To say that SIRP β1exGST or SIRP β1exIgG did not support cell adhesion was in many cases understating the case. In adhesion assay using rapidly-spreading cells such as fibroblasts, if the plate was left longer, the cells would begin to adhere even to the BSA blocking protein. This is not unusual in cells that secrete their own matrix proteins. In these cases SIRP β1ex

supported less adhesion even than the BSA blocking substrate. Although only a hint, searching for a role in cell migration might be promising for SIRP β. Since SIRP β may be a secreted protein under certain circumstances, and this would also support a role in chemotaxis.

The extracellular domains produced for use in adhesion assays also proved useful as antigens for immunisation. The secretion system yielded enough protein to construct affinity columns to purify specific antibody from the serum. An added advantage over bacterially produced GST fusion proteins of extracellular domains was that the antibody was raised against a eukaryotically-processed and glycosylated protein, and hence may recognise more physiologically-relevant epitopes. Considering the very high sequence identity between SIRP α1 and SIRP β1 in their extracellular domains, the antisera were very selective for their respective antigen. The fact that the antisera showed this selectivity is probably because if other aspects of the structure besides the primary peptide sequence, for example, different glycosylation patterns. Since glycosylation is not only a sequence-specific but also a tissue- and cell type-specific phenomenon, the variety in final structure may be wider *in vivo* than in the situation here, where the proteins were secreted from the same cell type (HEK293).

In conclusion to this section, it has been found that members of the SIRP family do have relevance to cell adhesion and adds a functional aspect to the structural relationship it has with the C-CAM members of the CEA superfamily.

6.2 Regulation of SIRP α by tyrosine kinases

The PP1 inhibition of SIRP α tyrosine phosphorylation induced by EGF and other ligands indicates that although SIRP α tyrosine phosphorylation is triggered by stimulation of a wide variety of different receptors, the pathway from ligand stimulation to SIRP α tyrosine phosphorylation is mediated by similar molecules. One of these is likely to be a src-family kinase, because PP1 is a src-family kinase inhibitor. Although most inhibitors exhibit a degree of non-specificity, and using inhibitors to identify specific proteins must be approached with caution, an inhibitor can very effectively eliminate a candidate, as in this experiment. Although

activation of the EGFR kinase activity certainly triggers SIRP α tyrosine phosphorylation, it is not inhibited by PP1. Because PP1 inhibits EGF-induced SIRP α tyrosine phosphorylation, the experiment eliminates the EGFR as the kinase that directly phosphorylates SIRP α.

Experimental evidence clearly shows here that the kinase or kinases that directly phosphorylate SIRP α are sensitive to PP1, a src-family kinase inhibitor, but that c-src and c-fyn are probably not the kinases that directly phosphorylate SIRP α in this system. On the contrary, overexpression of kinase-inactive c-src and c-fyn appear to induce tyrosine phosphorylation of SIRPs and this begs some explanation. It may be due to an adaptor function of src-family kinases. Kinase-independent functions of src-family kinases are known. Fgr, another src-family kinase, associates with SIRP α in macrophages and inhibits macrophage phagocytosis independently of its tyrosine kinase activity[86]. It is also mentioned that Fgr does not phosphorylate SIRP α and may therefore act as an adaptor molecule. Lck, another src-family tyrosine kinase enhances CD3-mediated signalling by a kinase-independent mechanism that may involve the SH2 domain of Lck [42] although the mechanism is unknown. All src-family kinases possess an SH2 domain so the adaptor function of src-family kinases in SIRP α tyrosine phosphorylation is feasible. A model would be that the src-family kinase recruits directly or indirectly another PP1-sensitive kinase that phosphorylates SIRP α, and downregulates its activity by phosphorylating an inhibitory residue on that molecule. Tyrosine kinases that are inactivated by tyrosine phosphorylation include the src-family kinases themselves [87], Syk[88,89] and possibly others. It is well-established that src-family kinases phosphorylate Syk, but instead of inhibiting Syk, they enhance its activity greatly.

Src-family kinases may also have a role in activating a phosphatase that is able to maintain SIRP α in a dephosphorylated condition. It also cannot be discounted that the kinase that phosphorylates SIRP α is not a src-family kinase but is still inhibited by PP1.

6.3 The SIRP β/DAP12/Syk multiprotein complex

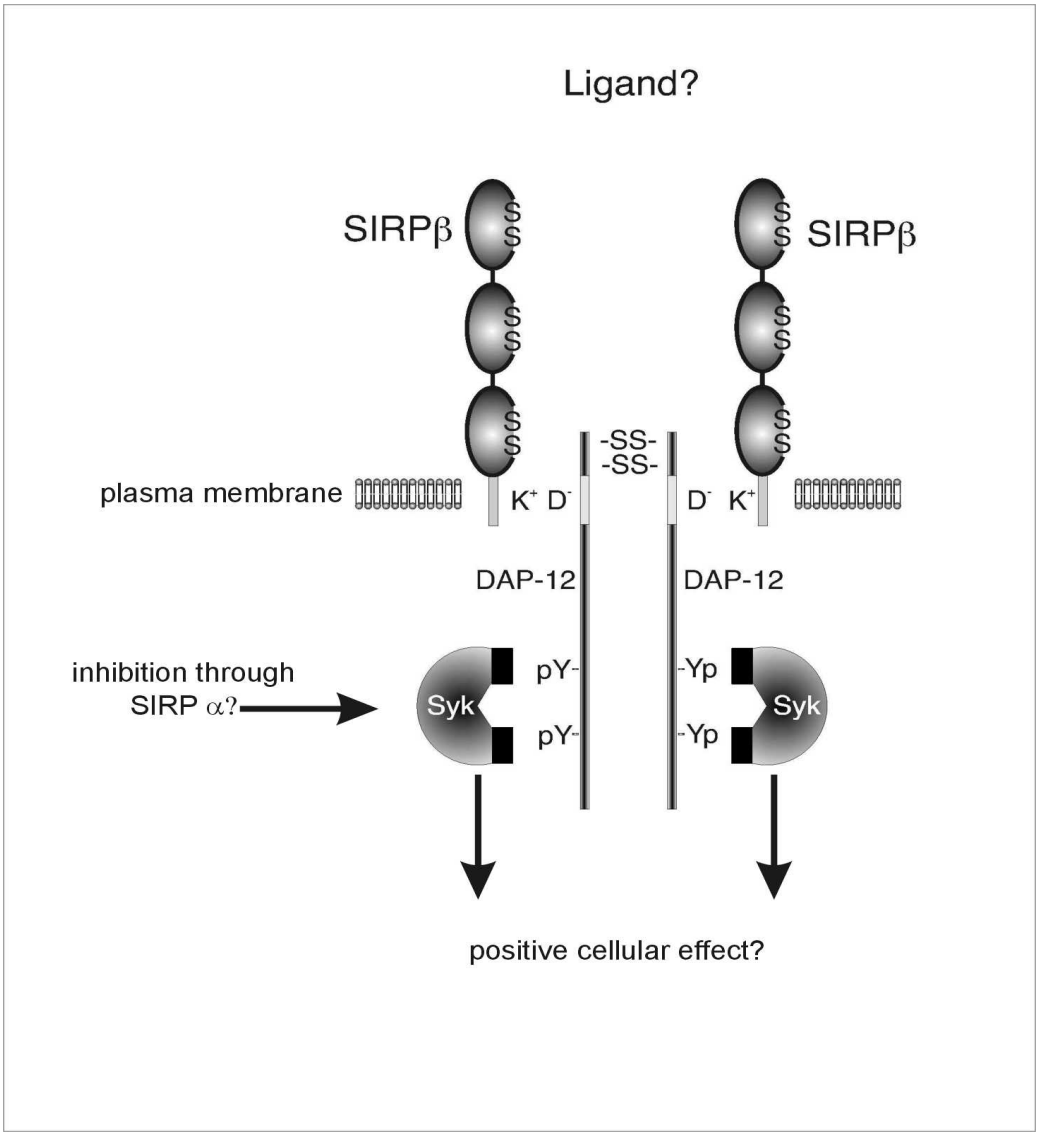


Figure 40 The SIRP β/DAP12 complex. SIRP β associates constitutively with the dimeric transmembrane adaptor protein DAP12 via an interaction in the transmembrane domain involving two oppositely charged amino acids. The tyrosine phosphorylated ITAM motifs of DAP12 associate with the SH2 domains of the cytosolic Syk tyrosine kinase. Signalling through this complex may have a positive influence on cellular survival. SIRP α may be able to inhibit signalling via this complex.

Evidence is provided here that SIRP β participates as a member of a multiprotein complex with DAP12 and that Syk is involved in that complex. Biochemical

evidence suggests that it may be a key mediator in signalling processes conducted via this complex. There is further evidence from studies with NIH3T3 cells overexpressing SIRP β/DAP12, that this complex is involved in the regulation of induced cell death, albeit through an as-yet unknown mechanism. With respect to the transformed cellular phenotype, SIRP β/DAP12 may here be in opposition to the inhibitory role ascribed to SIRP α.

6.3.1 Association of SIRP β with DAP12

The discovery of the association between SIRP β1 and DAP12 has placed SIRP β, within the emerging family of 'Inhibitory Activating Receptors (IARs)'[70 54]. This group of transmembrane receptors have structural similarity to SIRP β. These molecules have no intracellular domain and rely on the transmembrane association with another molecule for their ability to transduce a signal[3]. Two such adaptor molecules so far exist. These are DAP12[90], also known as KARAP[91-94], and DAP10 [95], which is also called KAP10[96]. The antigen receptor FcεRI, the ζ–chain of the CD3 complex and the Ig α/β heterodimeric subunits of the B-cell receptor (BCR) complex are also related to this pair of molecules[97].

DAP12 exists as a homodimer and possesses one ITAM motif[6,7,98] per monomer. Each ITAM motif contains two tyrosines that can be inducibly phosphorylated. Current opinion suggests that this is catalysed by a src-family kinase. Phosphorylation of both tyrosines in the ITAM provides a binding site for Syk, a cytosolic tyrosine kinase critical for antigen receptor signalling[99]. Both SIRP β1 and DAP12 have a charged amino acid in their transmembrane domain. SIRP β1 has a positively charged lysine, and DAP12 has an aspartic acid residue, that is negatively charged. This type of association was observed by Lanier in 1998 [90], where oppositely charged transmembrane amino acids were shown to be essential for the association between DAP12 and KIR2DS2, another IAR that is a member of the Killer Inhibitory Receptor(KIR) family[3,100]. NKG2D is another KIR that associates with DAP12[101]. SIRP β1 presumably interacts with DAP12 in a similar way.

Numerous molecules belonging to the SIRP β subtype have been discovered[1], and other molecules of the inhibitory receptor superfamily that resemble SIRP β in structure also associate with DAP12 [102] [103]. It has been reported that DAP12 is expressed ubiquitously in both haematopoietic and non-haematopoietic cells, particularly in epithelial and neural cells [93]. Northern blot analysis of DAP12 suggests that it has a more restricted expression than SIRP β, which is expressed over a broad spectrum of different cell lines (A. Ruschel and I. Sures, respectively, unpublished observations). This means that either there are other molecules similar to DAP12 forming complexes with SIRP β molecules, or SIRP β has a second role that it can perform alone. One such candidate molecule, DAP10 (also known as KAP10) [96] [95] has been discovered to associate with certain other IARs in a similar fashion to the SIRP β/DAP12 complex, but instead of an ITAM, DAP10 has a YXXM motif in its intracellular domain. The YXXM motif binds the SH2 domain of the p85 regulatory subunit of phosphoinositol-3 kinase (PI-3K). Two other molecules with similar characteristics, CD3ζ and FcεRI can be included with DAP12 and DAP10 to compose a small family. It is tempting to speculate that these proteins associate promiscuously with any IAR to produce a highly adaptable system of receptor generation. In this system the specificity for an extracellular ligand could be modified by the choice of the activating receptor subunit, while the signal transducing effect would be decided by the cytoplasmic proteins brought to the complex by the choice of DAP12-type receptor. Recent evidence suggests, however, that there is at least some degree of specificity generated by the transmembrane domain of the DAP12-type protein for its SIRP β-like activating receptor[101].

6.3.2 The Syk tyrosine kinase as a member of the SIRP β/DAP12 complex

The association of Syk to DAP12 potentially links the SIRP β/DAP12 complex to numerous established downstream signalling mechanisms. Syk has roles in many classical signal transduction pathways and is well studied through its involvement in B-cell receptor (BCR) complex signalling via the Igα/Igβ subunits.

Phosphorylation by the Syk kinase activates PLCγ directly, triggering calcium mobilisation through the second messenger IP_3 and activating NFκB via diacylglycerol (DAG). Syk also activates PI-3K, which, amongst many other effects, activates protein kinase B (or Akt), inhibiting apoptosis via the Bcl/Bax family. Syk associates to the multidomain adaptor protein Vav, which utilises Rho-family GTPases to control actin reorganisation, eliciting cell movement. Vav also modulates the p38 MAPK/JnK protein kinase pathway, and lastly the ERK/MAP kinase activation can also be affected by Syk activation of ras [104]. A complex that recruits and controls the activation of Syk therefore has the potential to regulate important cellular processes, such as cell activation, proliferation, movement and apoptosis.

Src-family kinases are thought to be involved in tyrosine phosphorylation of DAP12 and probably activation of Syk tyrosine kinase, for example Lyn is essential for full activation of Syk in B cell signalling through the BCR[104]. The complex of phosphorylated proteins that surrounds immunoprecipitated DAP12-GST is suggestive of an activating complex, and may be a sign that overexpression or more of the pathways listed above are being utilised.

6.3.3 Similarities between SIRP β/DAP12/Syk and SIRP α/SHP-2/1 complexes

Revealing similarities between Syk and the SHP-2 tyrosine phosphatase can be seen when they are compared. Each molecule exists in a family of two members with one other single, 'sister' molecule which is highly-related in sequence and structure: ZAP-70 in the case of Syk, and SHP-1 in the case of SHP-2. Although SHP-1 is present in some non-haematopoietic cell types, both ZAP-70 and SHP-1 are thought to be principally expressed in haematopoietic lineages, while Syk and SHP-2 are far more broadly distributed. Both Syk and SHP-2 are cytosolic molecules with two SH2 domains located at the N-terminal of the molecule followed by the catalytic region. Neither are constitutively localised at the membrane, but can be recruited to the membrane by intermolecular interactions with proteins containing phosphotyrosine-based motifs. Syk is recruited to ITAM

motif-containing proteins while SHP-2 is recruited to ITIM motif-containing proteins. Functional studies of SHP-2 are surprising, because when studied in isolation, it appears to have a positive role in signal transduction[37], while at the same time, its recruitment to the cell surface by SIRP α is essential for the inhibitory role conferred upon SIRP α[1]. Similarly, Syk has been long understood to play a leading part in cellular activation through immunoreceptors, however a recent paper reports its role as a tumour suppressor and inhibitor of cell proliferation in breast cancer[105]. It may be that these eccentric molecules confer either positive or negative roles, dependent upon their subcellular localisation. Lastly, when one compares the associated SIRP α/SHP-2 complex to that of SIRP β/DAP12/Syk, they look schematically similar, with the exception that the SIRP β/DAP12 complex is likely to be composed of two SIRP β and two DAP12 molecules, while SIRP α may only be a monomer. Since overexpression of SIRP α is able to inhibit the activity of Syk *in vitro*, it may be that the two complexes are intimately twinned, and it is tempting to propose their participation in a common mechanism, e.g. to modulate a signalling receptor complex, maybe localised to a raft.

6.4 Membrane translocation of SIRP β/DAP12 and stabilisation of expression

To discuss this section it is first necessary to be clear that there were two different levels of overexpression that produced different yet complementary effects. Rather than being contradictory to each other these effects are informative when the appropriate model is applied. When the pLXSN or pIRES vector system was used, the promoter that expressed the cDNA was the 5' LTR of the retrovirus. Although this overexpresses a desired construct, it is a much weaker promoter than the CMV promoter in the pCDNA3 vector. If a strong, CMV-driven expression system was used instead of a weaker one, not only would more protein be visible in the cell, but if it was trafficked to other areas in the cell, or even secreted, higher expression would favour its localisation closer to the source of protein synthesis. For SIRP β and DAP12 this would be the ER because they both have secretory signal peptides.

It is surprising to discover that overexpression of DAP12 at the plasma membrane requires co-overexpression of SIRP β, but remarkably, a search of the literature can find other similar examples amongst the immunoglobulin superfamily. Components of the pre-BCR complex are conditionally assembled in the ER before being transported to the plasma membrane. Reth (1990)[106] showed that the Ig α/Ig β heterodimeric component of the B-cell receptor complex is required for transport of the entire B-cell receptor complex to the surface of the cell. The T cell receptor complex is not expressed at the surface of the T-cell without coexpression of CD3ζ and *vice versa* [107]. Both CD3 ζ and Ig α/Ig β are similar to DAP12 because they are ITAM motif-containing transmembrane dimers with little extracellular domain.

Assisted DAP12 transport to the cell membrane has now been very convincingly proven by a daring expression cloning-style screen. Cells stably overexpressing an N-terminally epitope-tagged DAP12 were transfected with an expressed cDNA library and sorted for the presence of the tag at the plasma membrane surface. Sequencing the plasmids from the 'tag-positive' cells successfully pulled out SIRP β-type activating receptors from the library. SIRP β1 itself was among them[70].

6.4.1.1 Translocation of DAP12 to tyrosine kinases

Sodium pervanadate treatment inhibits endogenous tyrosine phosphatases surrounding DAP12. This induces DAP12 tyrosine phosphorylation that recruits endogenous Syk tyrosine kinase to DAP12. This is notably enhanced following overexpression of SIRP β1. Because SIRP β1 escorts DAP12 to the plasma membrane, the difference in *DAP12-GST* tyrosine phosphorylation after SIRP β1 overexpression is probably because of DAP12 translocation. This is therefore because SIRP β1 escorts DAP12 to the cell membrane where it comes into proximity with the kinases that are responsible for its activation. These kinases are therefore likely to be restricted to the cell membrane and not clustered around DAP12 before it reaches the membrane. Candidates would be the src-family

kinases, since they are permanently membrane-localised by myristoylation, and already implicated in the activation of other molecules that have ITAM motifs[88,108].

6.4.1.2 SIRP β as a potentially secreted protein

When SIRP β1 was overexpressed in NIH3T3 or HEK293 cells protein could be found in the cell culture medium. When DAP12 was expressed together with SIRP β1 the amount of SIRP β1 in the medium was depleted but was increased in the cell lysates. There is even more SIRP β in the medium when a stronger promoter system is used. If the amount of secreted SIRP β increases with expression, it is probably not proteolytically 'shedded'. If this were the case one would expect the sheddase activity to be overwhelmed by the substrate (SIRP β), and protein would accumulate at the plasma membrane. SIRP β-GFP is not seen in the membrane, so it either does not reach the membrane or it leaves the cell altogether. There are significant quantities of SIRP β in the supernatant when it is overexpressed in pCDNA3 meaning that it probably leaves the cell. Since it is hardly visible in the cell when expressed without DAP12 and under retroviral LTR control, it might mean that SIRP β has a second role as a secreted molecule in the absence of DAP12. If SIRP β really were a secreted protein, it would explain why it is difficult to detect in cell lysates in western blot when not overexpressed.

SIRP β1 cannot be detected in immunofluorescence at weaker LTR-driven expression when expressed alone, but when DAP12 is expressed SIRP β is clearly detectable at the plasma membrane. If SIRP β were secreted DAP12 would in this context be acting as a membrane anchor that would convert SIRP β from a secreted protein to one subunit of a transmembrane complex. Therefore DAP12 would modulate both the localisation and function of SIRP β. Interestingly, a SIRP β isoform has been recently found without the positively charged amino acid that would be essential for association with DAP12 [109]. In the absence of a membrane-anchoring coreceptor, this isoform may be targeted unconditionally to the supernatant.

If SIRP β molecules are potentially secreted, the SIRP family could be likened to semaphorins. This is a family of neuronal and haematopoietic receptors made up

of secreted forms as well as membrane-bound forms. The membrane bound forms in some cases have cytoplasmic domains that can participate in signal transduction. The secreted forms have been shown to have roles in axon guidance, with both chemoattractant and chemorepellent qualities. Semaphorins have also recently been discovered in haematopoietically-derived cells, and may also therefore have a role in lymphocyte adhesion/migration [110] [111]. The extreme unwillingness of the SIRP β1-GST fusion protein to act in cell adhesion, discussed earlier, may indicate a chemorepellent quality.

However, although it is tempting to speculate upon the possible role of SIRP β as a secreted chemoattractant (or indeed repellant), the hypothesis requires sturdy biological evidence to support it. Without this evidence, a role for SIRP β in the extracellular milieu cannot be addressed as more than a feasibility.

6.4.2 SIRP proteins in rafts, and/or caveolae

SIRP family members were found in fractions enriched with caveolin-1 that separated from plasma membrane fractions in ultracentrifugation experiments. Caveolae and rafts are structures in the plasma membrane that are unusually rich in cholesterol, and caveolin-1 is a marker protein for the caveolus. Caveolae are distinguishable from rafts so far only by the presence of caveolin-1. They were originally thought to be involved in clathrin-independent endo- or exocytosis, but after the techniques to purify rafts were improved, they were shown to contain a wide variety of proteins, many of them involved in signal transduction. Molecules of particular interest that have been found in caveolae include the src-family kinases, RTKs such as the EGFR, PDGFR and insulin receptors, G protein-coupled receptors, adaptor proteins such as SHC and Grb2, as well as PI-3K and MAP kinases. They are now thought to house, spatially organise, and even aid in the assembly of signal transduction complexes. They possibly also separate complexes from each other to avoid signalling chaos through unwanted cross-talk. Scientists studying the function of the trans-synaptic eph – ephrin signalling system used a raft model to propose that both halves of the complex on either side of the synapse are spatially arranged as though positioned on a platform.

Like SIRP molecules, the ephrins are a family of membrane proteins that have two subtypes. The A-type ephrins are fastened to the outside of the membrane by a GPI anchor and the B-type ephrins are transmembrane proteins. A-type ephrins are thought to associate with an as yet unknown transmembrane protein. This anonymous mediator recruits src-family kinases e.g. c-fyn to the A-type ephrin complex. The B-type ephrins have a cytoplasmic domain that binds tyrosine kinases when it is tyrosine phosphorylated. These associations are at least partly responsible for co-ordinating the cell adhesion and cytoskeletal rearrangement necessary for axon guidance.

The ligands for the A- and B- type ephrins on the surface of the neighbouring cell RTKs are the *eph* receptors, which are RTKs. The ephrins contact the ephs across the synapse and bring the ephs into sufficiently close contact with each other to activate them by transphosphorylation. This triggers a signal transduction cascade that motivates a chemotactic response by the other cell. Hence, a bi-directional signal transduction response is set up at a localised position at the cell surface.

Useful comparisons can be drawn between ephrins and SIRPs. SIRP α molecules are scaffold proteins residing at the membrane that can associate with certain receptors [23]and a variety of tyrosine kinases and adaptor proteins. SIRPs also exist in rafts, and SIRP α has at least one intercellular ligand. Although SIRP β is not known to be GPI linked, it is anchored to the membrane by another transmembrane protein that associates with Syk and maybe other tyrosine kinases. It may be possible to apply a similar model to the SIRPs in the future. A bi-directional SIRP α/CD47 signalling mechanism in the retina has already been reported[75].

The discovery of SIRP α in the raft fits quite well with its proposed role as a scaffold protein. One of its functions could be to recruit necessary proteins to the signal transduction complexes in the raft. One such a protein may be SHP-2, because its localisation is definitely influenced by SIRP α tyrosine phosphorylation. There may also be a role here for the different family members of SIRP α subtype in recruiting molecules to specific receptors. The single amino acid differences

between SIRPs might make them different enough to define the specificity of a receptor interaction.

6.5 Stimulation of the SIRP β/DAP12 complex with mAb B1D5C3

The mAb B1D5C3 antibody was used to activate the function of the SIRP β/DAP12 complex by SIRP β1 coligation. Although the antibody bound clearly to SIRP β1 at the cell surface, the treatment gave only a weak tyrosine phosphorylation of DAP12-GST that may even have been independent of the antibody. The possible reasons for this should be discussed. The affinity of the antibody for SIRP β1 is unlikely to be a reason, because it bound very quickly to the DAP12-GST complex. However, it may be that the antibody binds to an epitope that is present more than once on SIRP β1 and therefore is less efficient at coligating one SIRP β molecule with another. A weak coprecipitation of Syk with DAP12-GST was visible after one hour. This may mean that longer incubations might be necessary for better activation, giving the process a time frame that rather resembles the formation of a large cellular structure such as a focal adhesion than a typical receptor-ligand interaction.

Activation of signal transduction through the SIRP β/DAP12 complex through coligation has reported by other researchers. This activation resulted in MAPK activation and cellular activation in myeloid cells[70]. This data names SIRP β as an Inhibitory Activating Receptor (IAR) that opposes the inhibitory effect that SIRP α has on cell activation. Moreover it hints at a possible complementary relationship between SIRP α and SIRP β in the modulation of a signal.

6.6 Regulation of the SIRP β/DAP12 complex by SIRP α

Experiments presented here have suggested that SIRP α complexes with protein kinases both dependent on and independent of its own tyrosine phosphorylation. Evidence is also provided that the complex associated with SIRP α can inhibit SIRP β/DAP12/Syk activation, and that this may involve tyrosine kinases.

6.7 Kinases associated with SIRP α

Two tyrosine kinases are known to associate *in vitro* with SIRP α. These are CSK[20] and PYK2[23].

CSK is a protein tyrosine kinase that shares a strong degree of homology to the src-family tyrosine kinases, although it is not a membrane anchored protein and does not possess the c-terminal inhibitory tyrosine (Y527 in c-src). One function of CSK is, in fact, to inhibit src-family tyrosine kinases by phosphorylating their c-terminal tyrosine[42]. A conformational change caused by an intramolecular interaction between the SH2 domain of the src-kinase and this phosphotyrosine shuts down the kinase activity. The kinase activity that is dependent upon SIRP α tyrosine phosphorylation can phosphorylate the Lck c-terminal tyrosine so CSK is as a candidate for this activity.

PYK2 is a comparatively new tyrosine kinase that shares significant sequence homology with focal adhesion kinase (FAK). It is expressed prominently in haematopoietic cells and in the brain, fitting that of the SIRP family. While FAK plays an important role in signalling through integrins[112], the role of PYK2 is as yet not so clear. PYK2 associates to SIRP α independently of SIRP α tyrosine phosphorylation, although the nature of the association is not precisely characterised[22]. Enolase was phosphorylated by a tyrosine kinase activity independent of SIRP α tyrosine phosphorylation, but there are no reports of PYK2 being able to phosphorylate enolase, the other exogenous substrate used to detect kinase activity.

Enolase is an established substrate of src-family kinases often used in *in vitro* kinase assays, and therefore a src-family kinase may associate constitutively with SIRP α molecules. This could provide a role for the 'proline-rich' region near the C-terminal end of SIRP α because all src-family kinases possess an SH3 domain. Although the SH3 domain is involved in intramolecular interactions, it is displaced by proline-rich regions belonging to other molecules[87]. This suggests it could also have adaptor function. Src-family kinases have not yet been shown to be physically associated to SIRP α, but their involvement with SIRP α is often suggested.

6.7.1 Inhibition of SIRP β complex by SIRP α

Overexpression of SIRP α inhibited the activity of a molecule associated to DAP12 that was probably Syk. At the same time this molecule was phosphorylated. It suggests that SIRP α brings a kinase to the DAP12 complex that inhibits the signalling that is dependent upon Syk activity. Syk does not have a c-terminal inhibitory tyrosine in the style of a src-family tyrosine kinase, but src-family kinase activity is required for full activation of Syk in B cell activation through the BCR. If src-family kinases were involved in the activation of Syk bound to DAP12, src-family kinase inhibition would also inhibit Syk. CSK brought to DAP12 by SIRP α would therefore have the potential to inhibit Syk. There are also suggestions that CSK can inhibit the tyrosine phosphorylation of DAP12-GST. Since src-family kinases also likely phosphorylate DAP12, CSK may have a double role. The similar (albeit weaker) effect of PYK2 on DAP12 tyrosine phosphorylation in the same experiment is harder to explain but would also suggest that the wild type PYK2 has a negative effect on DAP12 tyrosine phosphorylation.

The structure of SIRP α closely resembles other inhibitory receptor superfamily members. Although the sequences are highly divergent between SIRPs and for example, KIRs, Fcγ receptors and other subfamilies, the ITIMs maintain their integrity. ITIM motif-containing Inhibitory receptor superfamily members have been shown to have suppressive effects on cell activation in cells from a haematopoietic lineage, primarily NK cells, T-cells and B-cells[2]. Where possible, these inhibitory effects were shown to be directed upon the positive signals generated by receptors containing ITAM motifs, for example the BCR Ig α and Ig β, CD3 ε, CD3 γ, TCR ζ, and Fc εRI γ. The ability of SIRP α to have a similar effect on ITAM containing receptors would appear to extend the issue. Coligation of a chimera containing the intracellular portion of SIRP α fused to the extracellular domain of the high affinity IgE receptor FcεRI inhibits tyrosine phosphorylation and cell activation through IgE stimulation of FcεRI. The chimera becomes tyrosine phosphorylated during coligation and binds SHP-1 and SHP-2 [113]. This means that SIRP α has the potential to deliver an inhibitory signal to complexes involving receptors with ITAM

motifs, and implies an ITIM-dependent mechanism relating SIRP α to other ITIM-containing receptors in haematopoietic cells.

6.8 Biological effects of SIRP β/DAP12 overexpression in NIH3T3 cells

Overexpression of SIRP β/DAP12 in NIH3T3 cells leads to an inhibition of induced cell death. The word 'apoptosis' is sparingly used because inter-histone DNA cleavage or caspase activation were not investigated. However, nuclear DNA visualisation using the fluorescent DAPI stain showed that TNFα induced nuclear condensation, and the cell death that results from TNFα treatment is generally accepted to be apoptosis and not necrosis.

The second phenotype produced by the SD469 cells was the increased rate of acid production by the cells as well as glucose uptake, both observations reflecting enhanced glycolysis. This is of interest, because resistance to apoptosis and enhanced glycolysis are two phenomena that are both hallmarks of cellular transformation and are characteristic of tumour cells.

Mechanisms leading to apoptosis are numerous and are better discussed elsewhere, but one relevant mechanism would be through PI-3K, because it is a substrate of Syk that is activated by tyrosine phosphorylation. Active PI-3K then activates Akt, a protein kinase which is an inhibitor of apoptosis thought to have effects upon the Bcl/Bax/Bad family of proteins[114]. Akt also leads to upregulation of the GLUT1,3 and 4 glucose transporters and activates PFK2. Both phenomena are factors that lead to enhanced glycolysis. Therefore, Akt activation may be one way by which both enchanced glycolysis and apoptosis resistance can be linked to SIRPβ/DAP12. However, experiments with PI-3K inhibition using Wortmannin were not able to show that either phenotype was dependent on PI-3K. Therefore, either the conditions of inhibition were not sufficient to properly inhibit PI-3K or the cells use different pathways to achieve the observed phenotype.

It would be interesting to know whether SIRP β/DAP12-mediated resistance to cell death and enhanced glycolysis are linked. Levitski [115,116] has noticed that inhibition of apoptosis and enhanced glycolysis are connected through src-family tyrosine

kinases. PP1 treatment at higher concentrations also inhibited the resistance to cell death in SD469 cells. Although the PP1 concentration used was too high to implicate src-family kinase activity, it would be interesting to know if PP1 also affected glucose uptake to link the two phenotypes mechanistically.

Therefore, two observations, a resistance of induced cell death and evidence of enhanced glycolysis in NIH3T3 cells overexpressing SIRP β/DAP12 in NIH3T3 cells have provided two hints that SIRP β/DAP12 may be factors that contribute to cell transformation. These proceed through as yet undefined pathways, but it would be too early to rule out Akt as a potential player and further experiments to define the mechanism might prove worthwhile.

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8 Summary

The SIRPs are a recently discovered family of glycoproteins comprising more than 30 members belonging to the immunoglobulin superfamily. The two different structural subtypes, termed SIRP α and SIRP β, are distinguished by the presence or absence of a cytoplasmic domain, respectively. SIRP α1, the first member of the family to be purified, had been characterised as a negative regulator of signal transduction, and transformation assays had suggested that it also had tumour suppressive effects. Little or nothing is known about the possible function of either the other SIRP α homologues or the members of the SIRP β subtype.

The Ig-like domains possessed in the extracellular domains of all SIRPs suggest they have binding partners outside the cell. Cell adhesion experiments using the extracellular domains of SIRP family members showed that SIRP α have adhesion molecule properties. This led to the identification of CD47 as one ligand for SIRP α, performed in collaboration with others[21] and confirmed here. Furthermore, these experiments suggested that SIRP α molecules have at least one further unknown ligand that is not CD47.

The discovery that SIRP α was a cell adhesion molecule with a regulatory role in signal transduction was expanded by *in vitro* kinase experiments and experiments with inhibitors of tyrosine kinases. They showed that SIRP α associated with more than one kinase activity, and that cytosolic tyrosine kinases, probably of the src-family, were necessary for SIRP α to regulate tyrosine phosphorylation of a receptor.

In contrast to SIRP α molecules, proteins belonging to the SIRP β subtype remain uncharacterised. Therefore a large part of this work concentrates on the SIRP β subtype, its associated proteins, localisation and possible function in a cell.

In vitro association experiments revealed that SIRP β is part of a multiprotein complex at the cell membrane, where SIRP β1 interacted with DAP12, an adaptor protein with a transmembrane domain. DAP12 linked SIRP β to a cytosolic tyrosine kinase identified as Syk confirmed by western blot and PCR from cDNA preparations of the cell lines used in these experiments. The interaction of Syk

with the complex required the tyrosine phosphorylation of DAP12. Coligating SIRP β molecules at the membrane with a SIRP β-specific monoclonal antibody recruited Syk to DAP12 where it could be activated by treatment with sodium pervanadate. *In vitro* kinase assays detected several unknown phosphorylated proteins associated with SIRP β/DAP12/Syk when Syk was activated that may represent signalling molecules operating downstream of the complex. Cotransfection experiments showed that SIRP α complexed with kinase activities that enabled it to inhibit both DAP12 tyrosine phosphorylation and Syk kinase activity. This suggested that both complexes at some point operated in close contact, so experiments were carried out to localise SIRP proteins in the cell.

Fractionation experiments discovered that SIRP α and possibly SIRP β could be detected in fractions that contained GPI microdomains, or caveolae. Similar investigations with the SIRP β1/DAP12 complex revealed that DAP12 was dependent upon SIRP β for its direction to the plasma membrane where it was activated by tyrosine kinases. Membrane localisation of SIRP β was similarly reliant upon DAP12 expression, however, further experiments suggested that SIRP β may be secreted from the cell in the absence of DAP12.

To address the potential role of SIRP β1/DAP12 complex in signal transduction, cell lines overexpressing SIRP β and DAP12 were analysed. Cell death assays suggested that the SIRP β1/DAP12 complex was a negative regulator of induced cell death, and that tyrosine kinases might be involved in this regulation. Cells overexpressing SIRP β and DAP12 showed an enhanced rate of acid production, corresponding to an enhanced rate of glucose metabolism. These observations suggests that, SIRP β1/DAP12 overexpression may be a factor that contributing to a transformed phenotype, works in opposition to SIRP α molecules.

This work views the SIRPs as components of a cluster of different proteins at the cell membrane that recruit and use other cytosolic proteins, among them tyrosine kinases and phosphatases. It shows that SIRP α molecules may collaborate with SIRP β family members to modulate the signals generated by other receptors in signal transduction. This modulation may influence aberrant cellular processes that lead to disease.

Publications arising from this work

Papers

1. Seiffert M, Brossart P, Cant C, Cella M, Colonna M, Brugger W, Kanz L, Ullrich A, Buhring HJ. Signal-regulatory protein alpha (SIRP alpha) but not SIRP beta is involved in T-cell activation, binds to CD47 with high affinity, and is expressed on immature CD34(+)CD38(-) hematopoietic cells. *Blood* **97**, 2741-9 (2001).

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Patents Published

1. Buehring, Hans-Joerg; Ullrich, Axel; Chen, Zhengjun; Cant, Charles. Antibodies against signal regulator proteins Int. Appl., 36 pp. Pub. no. DE10010616 A (2001).