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SH2 and SH3 Domain Interactions and Characterization of a Novel SH3-protein POSH2

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CONTENTS

4

1. ABSTRACT

Modular protein domains mediate protein-protein interactions in various cellular proteins, including adaptors, enzymes and scaffold proteins. Src homology 2 (SH2) domains comprise a relatively large group of domains, which mediate inter- and intramolecular interactions by binding to tyrosine-phosphorylated ligands. Phosphorylation of tyrosine residues in proteins controls many facets of signaling in multicellular organisms, and increased tyrosine phosphorylation is associated with uncontrolled cell growth. Another group of domains, Src homology 3 (SH3) domains, mediates protein interactions by binding to ligands containing a polyproline type II (PPII) helix. SH3 domains mediate generally relatively low specificity and affinity interactions, although this might be due to the use of short ligands lacking the regions flanking the binding interface of SH3 domains. These outside regions are known to contribute to the ligand binding of SH3 domains. POSH proteins, which have been implicated as scaffold proteins in the JNK-mediated apoptosis signaling cascade, contain SH3 domains. POSH proteins also participate in protein trafficking and the regulation of protein degradation via ubiquitination.

The importance of tyrosine phosphorylation for many biological processes has led to an interest in profiling global tyrosine phosphorylation in cells. However, many of the methods utilized are either not suitable for comprehensive phosphotyrosine profiling or have technical limitations regarding their applicability for high-throughput analysis. In our study, we exploited the fact that the tyrosine phosphorylation of proteins creates binding sites for SH2 domains. A far-Western approach and a newly developed reverse-phase Rosette assay were used to profile the tyrosine phosphorylation of selected ligands (platelet endothelial cell adhesion molecule-1; PECAM-1 and p21-activated kinase 2; PAK2) as well to assess global tyrosine-phosphorylation levels from cell lysates. SH2 domains exhibited different protein binding preferences, which reflected in their ability to recognize a specific subset of cellular proteins. Our methods elucidated SH2 interactions at the protein level and demonstrated increased binding of SH2 domains to adherent cells in addition to adhesion-specific SH2/ligand interactions.

The human genome was found to contain 296 different SH3 domains, and these domains were used in a phage display approach to determine which SH3 domains bound most strongly to our target proteins of interest: Nef, PAK2 and a disintegrin and metalloprotease 15 (ADAM15). An unbiased system for simultaneously assaying the complete human SH3 proteome was used to determine the preferred SH3/ligand interactions; this approach provided valuable information without the limitations caused by short peptide ligands or the skewing of variables caused by more indirect methods. Our approach identified both previously reported and novel SH3 domains that were capable of binding to the target proteins with nanomolar affinities. In addition to providing information regarding the SH3-mediated binding kinetics, this method also identified novel signaling proteins, such as the PAK2-binding scaffold protein plenty of SH3 domains 2 (POSH2).

POSH2 was found to be a highly homologous new member of the previously identified POSH family of proteins. POSH2 was shown to contain four SH3 domains and a RING domain, which provide ubiquitin E3 ligase activity to the protein. Activated Rac1, a GTPase, was shown to interact with POSH2, and this interaction was mediated by the partial Cdc42/Rac1 interactive binding (CRIB) domain. Moreover, the interaction of POSH2 and Rac1 suggests that POSH2 acts downstream of Rac1 in JNK-mediated apoptosis.

In conclusion, our results from the SH2 domain study provided information regarding likely *in vivo* interactions and changes in the concentration of SH2 binding sites under various conditions. Deciphering the global phosphotyrosine pattern and identifying activated signaling pathways are also important for understanding aberrant cellular functions. Thus, global phosphotyrosine profiling and quantification by SH2 domains could be used as a diagnostic tool in clinical applications. SH3 domains were suggested to have a more prominent role in mediating cellular interactions, and these domains bind with higher specificity than was previously anticipated. Moreover, the SH3 phage library system proved to be a valuable tool for deciphering the wiring of SH3-dependent signaling networks within the cell. POSH2 contains an SH3 domain and is the third member of the POSH protein family. These POSH proteins contain a highly homologous domain structure consisting of a RING finger domain, multiple SH3 domains and a partial CRIB domain, which is crucial for binding to Rac1.

2. LIST OF ORIGINAL COMMUNICATIONS

The thesis is based on the following communications, which are referred to in the text by their Roman numerals:

I   Machida, K, Thompson, CM, Dierck, K, Jablonowski, K, **Kärkkäinen, S**, Liu, B, Zhang, H, Nash, PD, Newman, DK, Nollau, P, Pawson, T, Renkema GH, Saksela K, Schiller, MR, Shin, D-G and Mayer BJ. High-throughput phosphotyrosine profiling using SH2 domains. Molecular Cell 2007;26:899-915.

II **Kärkkäinen, S**, Hiipakka, M, Wang, J-H, Kleino, I, Vähä-Jaakkola M, Renkema GH, Liss, M, Wagner, R and Saksela K. Identification of preferred protein interactions by phage-display of the human Src homology-3 proteome. EMBO Reports 2006;7:186-191.

III **Kärkkäinen, S**, van der Linden M and Renkema GH. POSH2 is a RING finger E3 ligase with GTPase binding activity through CRIB domain. FEBS Letters 2010; 584(18):3867-72

3. ABBREVIATIONS

ADAM	A disintegrin and metalloprotease
AIDS	Acquired immunodeficiency syndrome
AP	Alkaline phosphatase
AP-1	Activator protein-1
APS	Adapter with pleckstrin homology and Src homology domains
ArgBP	Arg binding protein
ASK	Apoptosis stimulating kinase
ATCC	American Type Culture Collection
BCAR1	Breast cancer anti-estrogen resistance 1 (protein)
BSA	Bovine serum albumin
Cbl	E3 ubiquitin protein-ligase/Casitas B-lineage lymphoma proto-onkogene
CRIB	Cdc42/Rac interactive binding domain
Csk	C-terminal Src kinase
Cten	C-terminal tensin-like (protein)
DLK	Dual leucine zipper-bearing kinase
DMEM	Dulbecco's modified Eagle's medium
DOCK180	Dedicator of cytokinesis 1 (protein of 180 kDa size)
ECL	Enhanced chemiluminescence
Eps8	Epidermal growth factor receptor pathway substrate 8
ERK	Extracellular signal-related kinase
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FCS	Fetal calf serum
GFP	Green fluorescent protein
Grb2	Growth factor receptor-bound protein 2
GSH	Glutathione
GST	Gluthathione S-transferase
HA	Hemagglutin
HEK	Human embryonic kidney (cell line)
HRP	Horseradish peroxidase
IVKA	*In vitro* kinase assay
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
Luc	Luciferase

KLB	Kinase lysis buffer
MAPK	Mitogen-activated protein kinase
MBP	Maltose binding protein
MEKK	MAPK/ERK kinase
MLK	Mixed-lineage kinase
Nef	Negative factor
p130Cas	130 kDa Crk-associated substrate (Cas)
PAK	p21-activated kinase
PBS	Phosphate buffered saline
PBT	Biotin purification tag
PDZ	PSD95/Discs Large/ZO-1 (domain)
PECAM	Platelet endothelial cell adhesion molecule
PH	Pleckstrin homology (domain)
PI3K	Phosphoinositide 3-kinase
PIX	PAK-interacting exchange factor
PJAC	POSH-JIP apoptotic complex
PLCC	Phospholipase C- γ1 (PLC-γ1) C-terminal SH2 domain
POSH	Plenty of SH3 domains
POSHER	POSH-eliminating RING protein
PPII	Polyproline type II (helix)
PSD-95	Post-synaptic density (protein) of 95 kDa
PTB	Phosphotyrosine-binding domain
pTyr	Phospho-Tyrosine
RING	Really Interesting New Gene (domain)
SAP	SLAM-associated protein
SLAM	Signaling lymphocytic activation molecule
SAPK	Stress-activated protein kinase
SEK	SAPK/Erk kinase
SH2	Src homology 2 (domain)
SH3	Src homology 3 (domain)
SHIP	SH2-containing inositol 5´-phosphatase
SHP	SH2-containing protein tyrosine phosphatase
SKAP55	Src kinase-associated phosphoprotein of 55 kDa
SOCS	Suppressors of cytokine signaling
STAT	Signal transducers and activators of transcription
SYF	Src, Yes and Fyn (cell line, deficient in these Src kinases)
TBS	Tris-buffered saline
Ubi	Ubiquitin
WB	Washing buffer
WISE	Whole interactome scanning experiment

10

4. INTRODUCTION

Proteins are composed of evolutionarily conserved, functional modules, which are called domains. These domains can mediate protein-protein interactions, which are important for transmitting information via signal transduction pathways and also for assembling multiprotein complexes. Therefore, domain interactions mediate many biological processes occurring within the cell. SH2 and SH3 domains were among the first modular domains identified, and both domain families consist of many members that can be found in various types of proteins. These domains participate in cell growth and differentiation, and thus, they are involved in many aberrant conditions such as oncogenesis and autoimmune diseases.

SH2 domains have a unique function in coupling proteins to cell-surface receptors via the recognition of tyrosine-phosphorylated ligands. Members of this large family of SH2 domains (approximately 120 SH2 domains are present in the human genome) have different preferences for binding to specific phosphotyrosines in ligands, which provides specificity for signal transduction. Increased tyrosine phosphorylation is associated with unregulated cell growth in cancer. The tyrosine phosphorylation profile of the cell varies depending on which tyrosine kinases are aberrant. In recent decades, the extensive studies on the various players in signal transduction pathways and the increased attention to high-throughput technologies for investigating global tyrosine phosphorylation, have provided valuable information on the impact of these proteins on tyrosine kinase signaling networks.

SH3 domains are another group of conserved protein folds. These domains are small (approximately 50-70 amino acids long) and recognize polyproline motifs that adopt the left-handed polyproline type II helical conformation. Most of the SH3 domain interactions are relatively weak and are generally thought to mediate interactions of low specificity. However, in addition to the core recognition motif, regions outside of the binding regions have been shown to provide specificity for the SH3 interaction. Following the discovery of SH3 domains more than two decades ago, the SH3 domain family has been expanding. Moreover, experimental

approaches have revealed the presence of these domains in many proteins involved in signaling and regulation, reflecting the importance of SH3 domains in cellular signaling.

SH3 domain-containing proteins include scaffold proteins, which enable the assembly of multiprotein complexes and the rapid flow of information between signaling proteins. The JNK signaling cascade is one of the signaling pathways that requires scaffold proteins to maintain the specificity of the signaling pathway. JNK signaling can mediate opposing cell fates such as apoptosis and cell survival; thus, the specificity and regulation of the signaling outcome is particularly important. POSH proteins are one group of scaffold proteins that play a role in the JNK signaling cascade. In addition to multiple SH3 domains, they contain a unique Rac GTPase binding domain, which links the POSH proteins to an upstream activator (Rac) of the JNK pathway. POSH proteins also have a RING domain, which possesses E3 ligase activity, enabling these POSH proteins to regulate turnover and trafficking of cellular proteins via ubiquitination.

The aim of this work was to identify interactions between SH2 and SH3 domains and novel ligands. In addition, we studied the phosphotyrosine binding properties of SH2 domains and their specificity for ligand recognition by using selected ligands of interest. Moreover, SH2 domain binding to tyrosine-phosphorylated ligands was investigated globally under various cellular conditions. We also identified SH3 domains in the human genome and used them to simultaneously determine which of the identified domains most strongly bound to our selected proteins of interest (Nef, PAK2 and ADAM15). Our goal was to characterize POSH2, a novel SH3 domain protein, which was found via a phage display screen of the SH3 domains. In addition, we studied the function of POSH2 and its role in JNK signaling.

12

5. REVIEW OF THE LITERATURE

5.1   Protein-protein interactions

Inter- and intramolecular communication is important for multicellular organisms. Signals from the environment control the behavior of a cell; these signals include direct cell-cell contacts and extracellular soluble factors such as growth factors, cytokines and hormones. These factors are often recognized by receptors, which convey the signal inside the cell. Diverse cell surface receptors recognize various signaling molecules by their extracellular domains. The binding of the factor to the receptor initiates signaling cascades, which terminate in the nucleus where specific genes are transcribed. The sum of these events determines the cellular response (Pawson and Nash, 2000).

Post-translational modifications such as phosphorylation and ubiquitination can regulate signal transduction events. Phosphorylation by protein kinases and the counteraction by phosphatases can regulate the activity of enzymes by changing their physical properties and can alter protein-protein interactions by creating or masking docking sites for other proteins. Phosphorylation is a highly dynamic and reversible process and provides a mechanism for the translocation of proteins within a cell. Ubiquitination, in which short ubiquitin proteins are attached to proteins, regulates protein degradation and can function as a signal for endocytosis (Deribe et al., 2010; Mayer, 2012; Scott and Pawson, 2009).

5.2   Small domains in signaling networks

Cellular behavior is regulated by the complex network of transiently interacting proteins. These proteins contain functional modular units, called domains, which determine the activity or binding properties of the protein (Scott and Pawson, 2009). When these small modular domains are isolated, they usually fold independently

(Pawson and Nash, 2003). Signaling domains are found for example in scaffold and adaptor proteins and kinases. Multidomain scaffold proteins organize the interacting proteins into a complex, thus enhancing the specificity and fidelity of signaling. (Scott and Pawson, 2009). Currently, more than 150 different signaling domains have been identified (http://smart.embl-heidelberg.de/). Some of these domains recognize proline-rich motifs (such as SH3 and WW domains), whereas others recognize for example phosphorylated tyrosine residues (such as SH2 and phosphotyrosine-binding (PTB) domains) on their ligands. Modular protein domains have enabled the evolution of specific cellular functions because domains can facilitate the formation of new complexes (by bringing proteins into close proximity that would not otherwise interact) and can regulate the intracellular localization of signaling proteins (Pawson and Nash, 2003; Yaffe, 2002). Reversible binding events between these modular domains and their cognate ligands are crucial for the formation of the controlled multiprotein networks.

5.3   SH2 domains

5.3.1   Discovery and occurrence of SH2 domains

Src homology 2 (SH2) domains are small protein folds (approximately 100 amino acids long), and they bind their ligands in a phosphotyrosine-dependent manner (reviewed in (Liu et al., 2012; Machida and Mayer, 2005; Pawson et al., 2004)). The human and mouse genomes contain 121 SH2 domains that are found in 111 proteins (Liu et al., 2006; Liu et al., 2011). However, the *Saccharomyces cerevisiae*, a unicellular fungus, contains only two SH2 domains and they recognize serine-phosphorylated ligands. Structural studies of these SH2 domains have suggested that they are ancestral forms of SH2 domain folds, mimicking phosphotyrosine recognition in mammals (Dengl et al., 2009; Diebold et al., 2010; Liu et al., 2011; Sun et al., 2010). The appearance of SH2-encoding genes in pre-metazoans and their subsequent expansion during the evolution of multicellular organisms; suggest that the structural ancestors of SH2 domains gave rise to the modern SH2 domains, which can mediate and meet the demands of complex phosphotyrosine (pTyr)-based signaling (Lim and Pawson, 2010). pTyr signaling is mediated by three functional

14

units: tyrosine kinases (which phosphorylate target tyrosines), phosphotyrosine phosphatases (which remove the phosphatases) and SH2 domains (which recognize the modifications) (see Figure 1A) (Lim and Pawson, 2010).
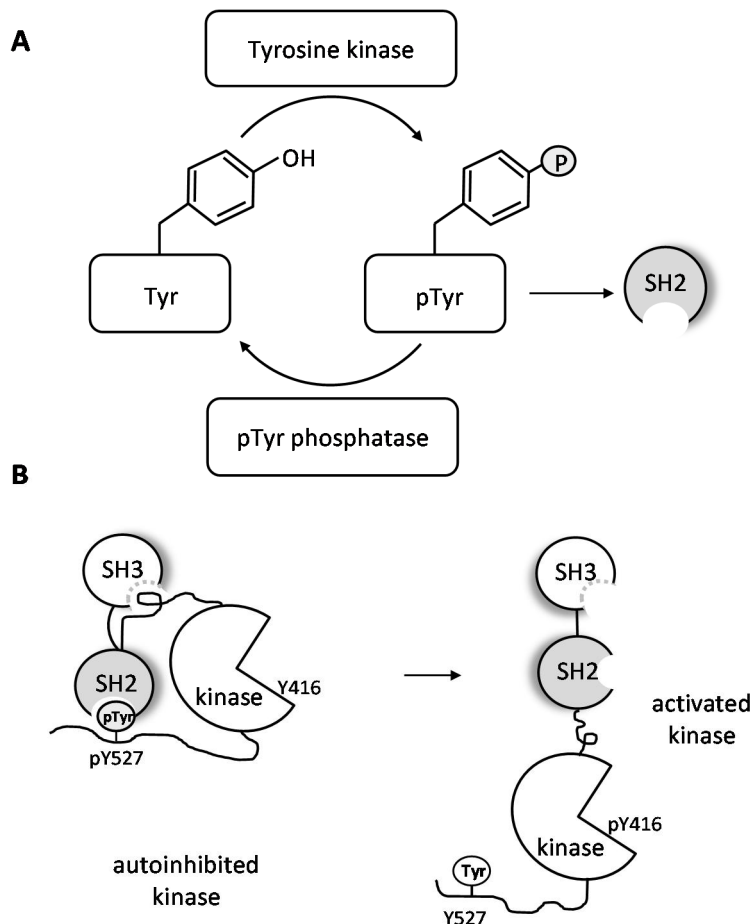
Figure 1. (**A**) Schematic overview of phosphotyrosine (pTyr)-based signaling. Tyrosine kinases, phosphotyrosine phosphatases and SH2 domains form a signaling platform for complex pTyr signaling. (**B**) SH2 domain regulates the activity of Src non-receptor tyrosine kinase in a pTyr-dependent manner. The left panel represents the inactive state of Src, and the kinase is in a "closed" conformation that is stabilized by the intramolecular interactions between the SH2 domain and pTyr527 and between the SH3 domain and the linker region (between SH2 and the kinase domain). The right panel represents the active, or "open", conformation of Src. Dephosphorylation of the inhibitory site (Y527) leads to enzyme activation and allows the binding of substrates to sites within the Src SH2 and SH3 domains.

SH2 domains were first identified as noncatalytic regions of similarity that were conserved in Src, Abl and Fps (also called Fes) oncoproteins (Sadowski et al., 1986). Because the SH2 domain did not possess catalytic activity and had a folded structure, this modular region was thought to function independently in different proteins. Some Fps mutations within this region influenced its transforming ability, suggesting that the SH2 domain played an important role in regulating Fps kinase activity (Sadowski et al., 1986). This domain was found to be capable of binding both activated receptor tyrosine kinases and cytoplasmic tyrosine-phosphorylated proteins (Mayer and Hanafusa, 1990; Moran et al., 1990). Thus, SH2 domains were implicated as mediators of efficient protein tyrosine kinase signaling.

Some proteins have dual SH2 domains, but other protein modules generally flank SH2 domains. SH2 domains often exist in proteins containing other protein binding domains (such as SH3, pleckstrin homology (PH) and phosphotyrosine-binding (PTB) domains) or catalytic domains (such as protein kinase domains) (Liu et al., 2011; Machida and Mayer, 2005; Pawson et al., 2001). SH2 domains are found in many cellular proteins, including adaptors and enzymes (Liu et al., 2006).

SH2 domains can control diverse cellular functions via their ability to regulate enzymes such as kinases and GTPases and processes such as ubiquitination and transcription. Therefore, SH2 domains are involved in cellular responses such as growth, survival and proliferation (Pawson et al., 2004). SH2 domains can also participate in trafficking proteins to specific sites within the cell (reviewed in (Pawson et al., 2001)). For example, adapter protein Grb2 (Growth factor receptor-bound protein 2) binds via its SH2 domain to an activated receptor tyrosine kinase and couples the phosphotyrosine signal to downstream targets. SH2 domains, together with other interaction modules (such as SH3 domains), can also regulate the activity of catalytic domains. Src nonreceptor tyrosine kinase is a classic example of this kind of regulation; the tyrosine kinase is maintained in an inactive conformation supported by SH2-SH3 intramolecular interactions (see Figure 1B) (Hubbard et al., 1998). Similarly, SH2-containing protein tyrosine phosphatase 2 (SHP2) is maintained in a repressed state via intramolecular interactions between its SH2 domain and phosphatase domain (Hof et al., 1998). SH2 proteins can also modulate signal transduction within a cell. Adaptor protein Cbl (E3 ubiquitin protein-ligase) binds in a pTyr-dependent manner to an activated receptor (such as a receptor tyrosine kinase) and non-receptor tyrosine kinases (such as Src tyrosine

16

kinase) and can promote their downregulation via ubiquitination (reviewed in (Machida and Mayer, 2005)). SH2-containing inositol 5-phosphatase 2 (SHIP2) controls its ubiquitination by using the SH2 domain to mask its ubiquitination site (De Schutter et al., 2009). Signal transducers and activators of transcription (STATs) are SH2-containing transcriptional regulators that link activated cytokine receptors at the cell membrane to the nucleus, where STATs regulate gene expression (Rawlings et al., 2004).

SH2 domain-containing proteins can also regulate the location and duration of receptor protein tyrosine kinase activity (Liu et al., 2006). For example, Cbl can promote the ubiquitination of the receptors in a phosphotyrosine-dependent manner, thus providing a recognition motif for the endocytic protein trafficking machinery (reviewed in (Haglund and Dikic, 2005)). Moreover, SH2 domain-containing proteins can also sustain the active state of the receptor, such as APS (Adapter with pleckstrin homology and Src homology domains), which SH2 domain interacts with the phosphorylated activation loop of an insulin receptor (Hu et al., 2003). Grb14, another SH2 domain-containing protein, also binds to the insulin receptor; however, its binding inhibits the activity of the receptor (Depetris et al., 2005).

The specificity of SH2 domain interaction with its cognate tyrosine-phosphorylated ligands is important for signaling fidelity. Mutations in SH2 domain-containing proteins have been described in many human diseases, including cancer, diabetes and immune disorders (Lappalainen et al., 2008; Liu et al., 2006).

5.3.2 Structure of an SH2 domain

The SH2 domain structure was solved in the early 1990's (Booker et al., 1992; Overduin et al., 1992; Waksman et al., 1992). The SH2 domain fold consists of seven β-strands (βA- βG), which form two antiparallel β-sheets and two α-helices (αA and αB) (Figure 2A). The two α-helices surround the central antiparallel β-sheet, which is formed by the βA-βC strands and the N-terminal βD and βG strands. A smaller β-sheet is composed of the C-terminal βD (often denoted βD´), βE- and βF-strands. The β-strands and α-helices are connected by loops, which are named by the letters of the secondary structural elements that they connect; for example, αA-helix and βB-strand are connected by the AB-loop. The core β-sheet divides the

domain into two functionally distinct sites, which form two ligand-binding pockets that recognize phosphotyrosine and mediate ligand specificity (Figure 2B). The N- and C-termini are in close proximity to each other and lie opposite of the ligand-binding site. This is characteristic of the modular nature of the domains and allows the protein to maintain its general structure (Pawson et al., 2002).

Approximately 120 SH2 domains have been analyzed and some variation in secondary structures has been observed in differences in loop lengths and in the conserved regions within the α-helices and β-sheets (Liu et al., 2006). Examples of atypical SH2 domains include the STAT, Janus kinase (JAK) and Cbl families, which possess different secondary structures between the central β-sheet and the C-terminus of the SH2 domain (see Figure 2C). Specifically, the Cbl SH2 domain lacks the smaller β-sheet (Meng et al., 1999).

Figure 2. (**A**) Structure of the SH2 domain. Reprinted from (Machida and Mayer, 2005) with permission from Elsevier. (**B**) The structure of Src kinase SH2 domain complexed with pYEEI tyrosyl phosphopeptide. Reprinted from (Bradshaw and Waksman, 2002), with permission from Elsevier. (**C**) Aligned sequences of SH2 domains discussed in the text. Secondary structural assignments are shown at the top of the figure. α helices are denoted in red, and β strands are denoted in blue. The conserved FLVRES sequence is outlined with a black box. Residues in the SH2 domain that coordinate phosphotyrosine are indicated with colored bars; conserved basic residues (Arg/Lys αA2, Arg βB5 and His βD4) are labeled with light red, acidic residues (Glu BC1) with blue, hydrophobic residues (Val/Leu βC) with gray and polar and uncharged residues (Ser βB7) with yellow. The N-terminal half of the SH2 domain is responsible for pTyr recognition, and the variable C-terminal half forms the specificity pocket. Modified from (Machida and Mayer, 2005) and (Liu et al., 2012).

A

B

pTyr-binding Region

Specificity Determining Region

pTyr, +1Glu, +2Glu, +3Ile

Structural labels (panel B): αA, βD, βC, CD, βE, βG, EF, BG, αB, βF, βE, DE, βD', BC

C

Structural element labels (top): βA | AA | αA | AB | βB | BC | βC | CD | βD | DE | βE | EF | βF | FB | αB | BG | βG

Sequence		Protein
WYFGKI---TRRESRLLLNAENPRGTFIVRES...TTK-GAYCLSVSDFDNAKGL------NVCHYKIRKLDSG-------GFYITSRT---GFN-SLQQLVAYYSKHADGLCHR----LITTV		Src
WYWGRL---SRQEAVALLQGQRH-GVFIVRDS...STSP-GDYVLSVSENS------RVSHYIINSSGPRPPVPPSPAQPPPGVSPSRLRIGDQE---F-D-SLPALLEFYKIHYLDTTLLIRP-VSR		Crk
WFFGKI---PRAKAREMLSKQRHD-GAFLIRES...ESAP-GDFSLSVKFGN------DVQHFKVLRDGAG-------KYPLWV---VKFN-SLNELVDYHRSTSVSR----NQQ		Grb2
WYYGKV---THQARNALNERGHE-GDFDIRDS...SSP-NDFSVSLKAQGK------NKHFKVQLKET--------VYCIGQRK---F--STMBELVEHYKKAPIFT---SEQG		Nck1
WFHPNI---TGVEAENLLLTRGVD-GSFLARPS...KSNP-GDFTLSVRRNG-----AVTHIKIQNTGD-------YYDLYGG---EKFA-TLAELVQYYMEHHQLKEK---NGD		SHP-2 (N)
VYHGKI---SRETGEKLLLATGLD-GSYLLRDS...SVP-GVYCLCVSHG------YIYTYRVSQTETG-------SWSAETAPGVHKRYFR-KIKNLISAFQKPDQGIVIP--LQYP		SAP
YYHGRI---TKQDCETLLLKEGVD-GNFLLRDS...SIP-GVLCLCVSFKN------VYRIQTIAEGSPKQVFP-SIKELISKFEKPNQGMVVH---LLKP		Eat-2
WFHGKI---TREQAERLLYPPET-GIFLVRES...TNYP-GDFTLCVSCDGK------VEHYRIMYHASK-------LSIDEE---VYFE-NDMQLVEHYTSDADGLCTR----LIKP		Csk
WYHRDL---SKAAAEELLARAGRD-GSFLVRDS...ESVA-GAFALCVLYQKH-----VHTYRILPDGED-------FLAVQTSQGVPVRRFQ-TLGELIGLYAQPNQGLVCA--LLLP		SHIP-2
THPGYMAFL-TYDEVKARLQKFIHKPGSYIFRLS...GTRL-GQWAIGYVTADG-----NILQTIPHNKP-------LFQALIDGFRBGFYL--------FPD		Cbl
WNEGYIMGFISKERERAILSTKPP-GTFLLRFS...SSKEGGVTFTWVEKDISGKTQIQSVEPYTKQQLLNNMSFAEITMG-------YKIMDAT---NILV-SPLVYLYPDIKESAF		STAT3

Structural element labels (bottom): βA | AA | αA | AB | βB | BC | βC | CD | βD | DE | βE | EF | βF | FB | αB | BG | βG

pTyr-binding region residues

Specificity-determining region residues

5.3.3 SH2 / ligand binding site and the binding properties of SH2 domains

The ligand binding surface within the SH2 domain is relatively flat and contains two discrete binding regions (Figure 2B), which are located on either side of the core β-sheet (Eck et al., 1993; Waksman et al., 1992). The "phosphotyrosine (pTyr)-binding region" is formed by the side chains of positively charged amino acids. The second binding region, or "specificity determining region", is more variable and this region is responsible for selectively interacting with ligand residues on the C-terminal side of pTyr.

Phosphate recognition is conducted by several residues, and arginine (Arg βB5, where β denotes the secondary structural element and B5 the position of the residue in the β-strand) is highly conserved and is often present as part of the FLVRES-sequence motif (see Figure 2C) (Waksman et al., 1992). This invariant arginine forms bidentate hydrogen bonds with the phosphate group of the pTyr. In addition, the pTyr-binding area is flanked by conserved residues within αA (Arg/Lys αA2) and βD (His βD4) structures; these residues interact with the pTyr phenol ring (Waksman et al., 1992). These interactions provide the basis for ligand binding and are characteristic of SH2 domains. Moreover, mutation of either the critical Arg βB5 or His βD4 abolishes pTyr-peptide binding (Bibbins et al., 1993; Bradshaw et al., 1999; Marengere and Pawson, 1992; Mayer et al., 1992).

The second binding pocket, the specificity-determining region, is defined by the βD and βE strands and the BG and EF loops (Eck et al., 1993; Waksman et al., 1993). This region has an extended surface, which binds residues C-terminal to pTyr and provides the ligand binding specificity (reviewed in (Bradshaw and Waksman, 2002; Eck et al., 1993; Pawson et al., 2001)).

5.3.3.1 *Ligand motifs recognized by SH2 domains*

Early studies of SH2 domains demonstrated that the domain binds to tyrosine-phosphorylated proteins (Mayer et al., 1991). The phosphotyrosine-containing motif contains the pTyr-x-x-x sequence, where x denotes the three residues C-terminal to the pTyr within the phosphopeptide sequence. Structural analysis of the Src and Lck SH2/ligand complexes (Eck et al., 1993; Waksman et al., 1993) revealed that the

pTyr-containing peptide adopts an extended conformation and binds perpendicularly to the core β-sheet, enabling the ligand to interact with phosphotyrosine in the first pocket and the hydrophobic residue C-terminal to pTyr (pTyr+3) in the deep cavity. Therefore, the typical SH2-binding model has been likened to "a two-pronged plug engaging a two-holed socket", where the side chains of the pTyr and pTyr+3 residues accommodate the deep pockets of the SH2 domain surface (Waksman et al., 1993). However, a pTyr-dependent recognition mode should dominate, enabling the SH2 domain to act as a phosphorylation-dependent binding module (Bradshaw et al., 1999). The Src SH2 domain bound to a pTyr-containing peptide sequence with 1000-fold higher affinity than a non-phosphorylated form of the same peptide (Bradshaw et al., 1999).

The ability of SH2 domain to preferentially bind specific tyrosine-phosphorylated proteins depends on its capability to recognize different phosphopeptides. The residues adjacent to the pTyr dictate the binding specificity of the SH2 domain, just as 'a zip code specifies an address' for mail delivery (Songyang and Cantley, 2004). Determining the binding specificity of SH2 domains provides information on potential *in vivo* binding partners and biological outputs. Furthermore, this information will be useful for designing inhibitors to be used therapeutically (Machida and Mayer, 2005).

The first studies were conducted using a phosphopeptide library to identify preferred SH2 binding partners (Songyang et al., 1993). Src family SH2 domains bound specifically to a pTyr-peptide containing a glutamate-glutamate-isoleucine sequence (pYEEI sequence) immediately C-terminal to pTyr at positions +1, +2 and +3, respectively (Songyang et al., 1993).

5.3.3.2   *Different ligand binding modes of SH2 domains / unusual SH2 domain interactions*

Many SH2 domains bind an extended phosphopeptide, which crosses the core β sheet and presents at least three C-terminal residues to the specificity-binding pocket. However, SH2 domains are notably versatile with respect to ligand binding due to the differences in the SH2 domain structure that prevents the customary pattern of binding. Firstly, some SH2 domains bind extended binding motifs.

Secondly, the SH2/ligand interaction can be independent of the Tyr-phosphorylation status. Finally, complex interactions may not conform to typical ligand recognition modes.

Because of the residue variability in the BG and EF loops, which are responsible for interacting with the residues that are C-terminal to the phosphotyrosine, different binding preferences for SH2 domains have been described (Pawson and Nash, 2000; Songyang et al., 1993; Waksman et al., 1993). Some SH2 domains have been reported to bind longer phosphopeptides by recognizing both N- and C-terminal residues relative to pTyr. The phospholipase C-γ1 (PLC-γ1) C-terminal SH2 domain (PLCC) contains an extended peptide-binding interface, which extends the contact region to the C-terminal +6 residue with respect to pTyr (Pascal et al., 1994). Similarly, the Cbl SH2 domain binds to its interaction partner, APS, by extending the contact region to the N-terminal -6 residue with respect to pTyr (Hu and Hubbard, 2005). Moreover, the signaling lymphocytic activation molecule (SLAM)-associated protein (Sap; also called SH2D1A or DSHP) SH2 domain interacts with ligand residues amino- and carboxy-terminal to the crucial tyrosine (Poy et al., 1999).

Classically, the SH2 domain binds to the specific ligand sequence in a pTyr-dependent manner. However, some SH2 domains, such as Sap and Eat-2 (also called SH2D1B), share similar binding preferences with non-phosphorylated peptides; the affinity of this interaction is only slightly weaker compared to the affinity for the Tyr-phosphorylated peptide (Li et al., 1999; Poy et al., 1999).

Complex interactions may involve features of the canonical recognition mode, but subtle differences in the ligand-binding interface can alter the binding mode. Commonly, SH2 domains bind ligands in an extended conformation. However, the Grb2 adapter protein binds ligands containing the pYxN-peptide (where x denotes any amino acid); these ligands adopt a β turn conformation (Rahuel et al., 1996). Here, a bulky tryptophan residue in the EF1 loop of Grb2 closes the pY-binding cleft and forces the phosphopeptide to turn away from the SH2 domain surface, leaving the pY+2 position available for the domain-ligand interaction. The SH2 domain-containing APS adaptor protein binds the Tyr-phosphorylated insulin receptor. The pTyr-binding pocket within the APS SH2 domain is occupied conventionally by pTyr, but the activation loop within the insulin receptor kinase

domain runs parallel with the core β sheet and positions another pTyr at the pY+4 position (Hu et al., 2003).

SH2 domains can act as adaptors by binding multiple ligands via other regions in addition its pTyr-binding motif, thereby linking pTyr-ligands to SH3-containing proteins. The Crk SH2 domain has been reported to contain a secondary binding surface, the PxxP motif, which binds to the Abl SH3 domain (Donaldson et al., 2002). Additionally, the Sap SH2 domain interacts conventionally with the SLAM receptor but engages the SH3 domain of FynT via an additional binding surface in the SH2 domain (see Figure 3A) (Chan et al., 2003; Latour et al., 2003). This activates Fyn kinase, which phosphorylates tyrosines in the cytoplasmic region of the SLAM receptor, thereby recruiting SH2 domain proteins that modulate T cell activation (Latour et al., 2003). Moreover, SH2 domain repeats (two SH2 domains in tandem) allow for increased binding to phosphotyrosine motifs containing several pTyr residues and thus provide greater ligand binding specificity (Yaffe, 2002).

The canonical SH2 domain typically recognizes residues C-terminal to the pTyr. The SH2 domain of c-Cbl can also bind peptides in the reverse orientation by positioning the N-terminus of the phosphopeptide near the C-terminal specificity pocket of the c-Cbl SH2 domain (Ng et al., 2008). Moreover, this is the first described SH2 domain that has the capacity to bind to ligands in two orientations (Ng et al., 2008).

5.3.4 Tyrosine phosphorylation and SH2 domain binding in cellular processes

Tyrosine phosphorylation is a post-translational modification that regulates many biological processes, such as cell adhesion, motility, differentiation, proliferation and apoptosis (Hunter, 2000; Lemmon and Schlessinger, 2010). The tyrosine-phosphorylated protein levels are maintained at low levels in resting cells via the regulation of both transmembrane receptor tyrosine kinases and cytoplasmic non-receptor tyrosine kinases. Both of these kinases are responsible for directly modifying of the tyrosine residues by transferring a phosphate group from ATP to a substrate. However, aberrant tyrosine phosphorylation has been detected under some conditions, such as in cancer (Blume-Jensen and Hunter, 2001; Hanahan and Weinberg, 2011; Krause and Van Etten, 2005). Increased tyrosine phosphorylation

during physiological processes as well under aberrant conditions creates more binding sites for cytoplasmic proteins containing SH2 and PTB domains (Pawson, 2004).
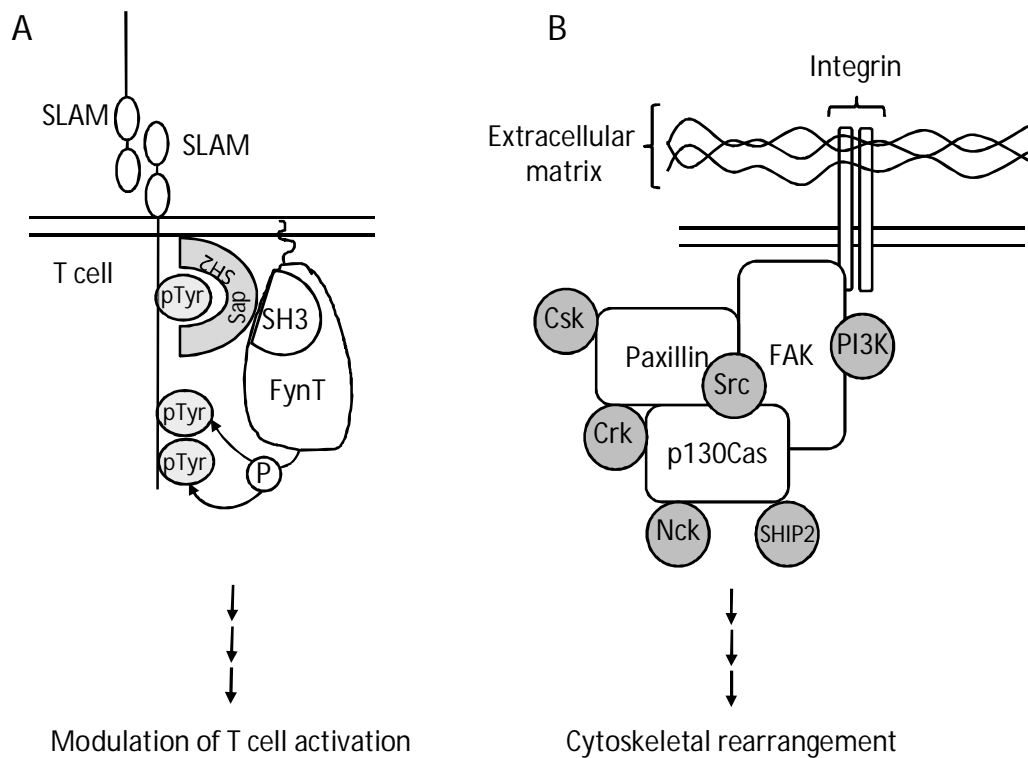
Figure 3. Examples of SH2-containing proteins in signal transduction pathways. (**A**) The SH2-containing Sap protein regulates SLAM-mediated (SLAM; signaling lymphocytic activation molecule) immune receptor signaling. In T cells, Sap binds to Tyr-phosphorylated SLAM following the receptor engagement and facilitates recruitment of non-receptor Src-family kinase Fyn (via Fyn SH3 domain). Interaction between the Sap SH2 and Fyn SH3 domains leads to Fyn activation and the subsequent phosphorylation of SLAM. Phosphorylated tyrosines serve as docking sites for SH2 domain-containing downstream proteins that modulate T cell activation. Modified from (Machida and Mayer, 2005). (**B**) A simplified schematic model of a focal adhesion signaling complex, in which SH2-domain-containing proteins (shown in gray) mediate signal transduction from integrins to the cytoskeleton.

5.3.4.1   *Examples of SH2 binding proteins in cell adhesion*

Cell adhesion is a fundamental mechanism that controls the behavior of a cell. Adhesion is important for the proliferation of adhesion-dependent cells, such as fibroblasts and epithelial cells. Normal cells are dependent on attachment to solid substrates for their survival and proliferation, but tumor cells can thrive independent of surface attachment (Hanahan and Weinberg, 2011). Cells adhere to the extracellular matrix through members of the transmembrane protein family of integrins, which elicit activation of downstream signaling and indirectly promote tyrosine phosphorylation of many cytoskeletal and signaling proteins by serving as an assembly platform for signaling complexes (Giancotti and Tarone, 2003). Many non-receptor protein tyrosine kinases (e.g. Src family kinases) are activated upon cell adhesion (Giancotti and Tarone, 2003). Cells lacking Src, Yes and Fyn kinases (SYF cells) have shown dramatically decreased protein phosphotyrosine levels, suggesting a major role for these kinases in global tyrosine phosphorylation (Klinghoffer et al., 1999). Moreover, Src kinase directly binds different focal adhesion proteins, such as focal adhesion kinase (FAK) and paxillin, via its SH2 domain (Cary et al., 2002). Additional SH2 domain interactions with FAK and paxillin have been described, including FAK/PI3K and paxillin/C-terminal Src kinase (Csk) (Chen et al., 1996; Sabe et al., 1994). FAK tyrosine kinase has been reported to phosphorylate Crk-associated substrate (p130Cas/also called breast cancer anti-estrogen resistance 1; BCAR1) in the focal adhesion protein complex, thereby creating binding sites for SH2 domain-containing proteins such as Crk, Nck and SHIP2 (Prasad et al., 2001; Schlaepfer et al., 1997; Vuori et al., 1996). The Crk adaptor protein forms multi-protein complexes via its protein interaction domains (Feller, 2001). Crk binds to p130Cas and paxillin proteins via its SH2 domain, and to DOCK180 (also known as dedicator of cytokinesis 1) and C3G (a guanine nucleotide exchange factor) via its SH3 domain. This multi-protein complex regulates small GTPases participating in cell adhesion (see Figure 3B).

Platelet endothelial cell adhesion molecule-1 (PECAM-1, also known as CD31) belongs to the immunoglobulin superfamily of cell adhesion molecules and is expressed in leukocytes, platelets and epithelial cells (Newman and Newman, 2003). It is a transmembrane protein, which contains a cytoplasmic domain containing two potential tyrosine phosphorylation sites. These tyrosines (Y663 and Y686) are

located within the immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which form part of the modular system that determines the cellular responses (Newman, 1999). PECAM-1 ITIM motifs, which conform the prototypical ITIM sequence ((Ile/Val/Leu/Ser)-x-Tyr-x-x-(Leu/Val)) and function as binding sites for SH2 domains following tyrosine phosphorylation. Src and Fyn SH2 domains have been shown to interact with tyrosine-phosphorylated PECAM-1 (Lu et al., 1997; Masuda et al., 1997). Interactions between PECAM-1 and the SH2 domain-containing Csk has been proposed (Newman and Newman, 2003) and the Sap/PECAM-1 interaction was recently confirmed (Quiroga et al., 2007). In addition, SH2 domain-containing PLC-γ1, SHP-1, SHP-2 and SHIP interact with tyrosine-phosphorylated PECAM-1 (Pumphrey et al., 1999). SHP-2, which contains tandem SH2 domains, was found to bind pY663 via its N-terminal SH2 domain. Moreover, tandem SHP-2 SH2 domains promoted cooperativity and enabled more efficient binding of SHP-2 to both pY663 and pY686 in PECAM-1 (Pumphrey et al., 1999).

5.3.5 A scheme for different screening approaches

Protein tyrosine kinases, protein tyrosine phosphatases, and their substrates play important roles in regulating normal cellular behavior and are also involved in aberrant cellular behavior (Hunter, 2000). Profiling the global tyrosine phosphorylation state in cells under different conditions is important for understanding *in vivo* interactions and signaling pathway activities. Therefore, profiling the tyrosine phosphoproteome could be the starting point for molecular diagnostic approaches. Tyrosine phosphorylation creates binding sites for SH2 domains, and this guided the development of various tools to investigate global tyrosine phosphorylation levels and to characterize the SH2 binding profile. These approaches could be categorized as identification-based (such as mass spectrometry-based) and detection-based methods (which exploits for example the potential of phospho-specific antibodies) (Machida and Mayer, 2005).

Several studies have characterized the phosphoproteome using sensitive mass spectrometry-based approaches (Kim et al., 2005; Olsen et al., 2006). These approaches enable identification of phosphoproteins but usually require large amount of sample and enrichment of the phosphoproteins. Moreover, mass

spectrometry-based methods are not sensitive enough for routinely detecting tyrosine-phosphorylated proteins, which are of low abundance in cells (Machida et al., 2003; Olsen et al., 2006).

Some studies have exploited the potential of antibodies, which can recognize specific phosphorylated sites. Although these methods may not identify the phosphoprotein, they may be useful for high-throughput and sensitive applications. Forward-phase assays, in which the phospho-specific antibodies are immobilized on a substratum, and are probed with the analyte, have been used to monitor the activation of the EGF receptor in different tumor cell lines (Nielsen et al., 2003). Reverse-phase assays utilize phospho-specific antibodies to detect immobilized protein samples that are spotted on an array, and these assays have been used to profile signal transduction pathways during various immunological responses and during disease progression (Chan et al., 2004; Paweletz et al., 2001). Paweletz and colleagues utilized a reverse-phase protein array approach, in which immobilized human cells were probed with phospho-specific antibodies to investigate cancer progression (Paweletz et al., 2001). Here, changes in phosphorylation status of selected signaling proteins correlated with cancer progression, suggesting that this method could be applied to monitor proteomic changes in patient tissue samples in a high-throughput format. Although profiling tyrosine phosphorylation using phosphotyrosine-specific antibodies provides valuable information on the phosphorylation status of cells, the number of phospho-specific antibodies is relatively limited. This type of approach is also biased because of the present (and limited) knowledge of important pathways and the proteins that are known to be involved in these pathways; thus comprehensive profiling of tyrosine phosphorylation is not achieved (Machida et al., 2003).

Videlock and colleagues used a phage display method to screen phosphopeptides against a human cDNA phage display library (Videlock et al., 2004). The authors could select nine high-affinity peptide binders, which encoded distinct SH2 domains (Videlock et al., 2004). Different approaches have been developed to profile SH2 binding sites in cells. Jones and colleagues developed a forward-phase assay using protein microarrays containing a nearly complete set of human SH2 and PTB domains (Jones et al., 2006). They produced approximately 160 SH2 and PTB domains and immobilized them on microarrays to analyze the binding of these domains to fluorescent phosphotyrosine peptides (corresponding to potential EGF

receptor family tyrosine phosphorylation sites). The comprehensive microarray approach allowed them to quantitatively analyze 5247 interactions and to construct a protein-interaction network via a computational approach. This network provided insights into the recruitment of proteins to the receptor by accounting for different binding affinities. The data from this study resulted in the identification of new interactions and confirmed previously recognized interactions, further suggesting that this method could be used to discover potential interactions between purified peptides and domains.

To assess SH2 binding to phosphoproteins in a cell lysate, Yaoi and colleagues developed a forward-phase bead-based assay, in which various SH2 domains were conjugated to microspheres containing fluorophore labels (Yaoi et al., 2006). Compared to common pull-down assays, one advantage of this format is that different bead-coupled SH2 domains can compete with each other for the same target proteins in the cell lysate enabling each SH2 domain binding profile to be simultaneously determined. The authors used this assay to profile the response of different cell lines to EGF treatment and they identified distinct SH2 binding patterns (Yaoi et al., 2006). Because adequate methods (utilizing SH2 domains) to quantitatively analyze low levels of tyrosine-phosphorylated proteins were lacking, Dierck and colleagues developed a new proteomic approach (Dierck et al., 2006). This reverse-phase multiplexed binding assay was based on labeling different SH2 domains with specific oligonucleotide tags. The assay allows competitive binding of tagged SH2 domains to protein mixtures, and the specific interactions are detected by using sensitive PCR amplification. This method was used to profile patient leukemia samples, and distinct binding preferences could be distinguished for 10 SH2 domains in different types of leukemia (Dierck et al., 2006).

These SH2 profiling methods provide valuable information regarding SH2/ligand interactions and global tyrosine phosphorylation state in the cell, but many of these methods lack adequate sensitivity complicating the collection of reliable quantitative binding data. The lack of adequate sensitivity is a particularly relevant obstacle when analyzing whole cell lysates, where the amount of tyrosine-phosphorylated proteins is very small. Limited amounts of phosphoproteins make forward-phase assays (in which SH2 domains are immobilized onto a substratum) less suitable for analyzing crude cell lysates when compared to reverse-phase assays. In addition,

many of these profiling methods involve expensive and specialized instruments, which are not accessible to many investigators (Machida et al., 2003).

5.4   SH3 domains

Src homology 3 (SH3) domains are small protein folds (50-70 amino acids long) and are characterized by their ability to bind to proline-rich peptide modules (reviewed in (Hiipakka, M., 2005; Mayer, B.J. and Saksela, K., 2004; Mayer, 2001; Musacchio, 2002)). The SH3 domain is one of the most commonly found modules in eukaryotic genomes. This region of similarity is present in many proteins, including 25 in *S. cerevisiae* (Mirey et al., 2005); these proteins contain at least one copy of the SH3 domain. The SH3-like fold is an ancient protein fold and is also found in prokaryotes (Whisstock and Lesk, 1999).

SH3 domains were first identified as independent regions of sequence similarity between the N-terminus of the Src family of non-receptor tyrosine kinases, phospholipase Cγ (PLCγ) and the Crk oncogene (Mayer et al., 1988; Stahl et al., 1988). Like SH2 domains, this small domain was recognized in different proteins and had no apparent enzymatic activity, implying that the domain was modular and possessed independent functions in protein complexes. These domains are found in many cellular proteins, including scaffold proteins, adaptors and enzymes.

SH3 domains can regulate different cellular functions (Kaneko et al., 2008; Mayer, 2001). These domains are important for intramolecular interactions that can regulate the activity of kinases. Classical examples of these intramolecular interactions include Src kinases and mixed lineage kinases (MLKs), which are normally maintained in an inactive conformation (Hubbard et al., 1998; Zhang and Gallo, 2001). Scaffold proteins, such as Plenty of SH3s (POSHs), contain multiple SH3 domains that mediate the assembly of multi-component signaling complexes by providing docking sites for these intermolecular contacts (Tapon et al., 1998; Wilhelm et al., 2007a). SH3 domains can contribute to the subcellular localization of some proteins. Adaptor proteins such as Grb2 and Nck consist of only SH3 and SH2 modular domains, and these proteins mediate the recruitment of p21-activated kinases (PAKs) to the membrane. SH3 domains have also been implicated in

cytoskeletal organization (Buday et al., 2002; Kioka et al., 2002) and endocytosis (McPherson, 1999).

5.4.1 Structure of an SH3 domain

The SH3 domain structure was solved about 20 years ago (Musacchio et al., 1992; Noble et al., 1993; Yu et al., 1992) and showed a conserved fold (Figure 4A). The SH3 domain fold is composed of five β-strands (β_A-β_E) and a single turn of a 3_{10} helix. The SH3 domain is formed by two β-sheets that are perpendicular to one another. The first sheet is formed by antiparallel runs of β_A, half of the β_B and the β_E, while the second sheet is composed of the second half of the β_B, β_C and β_D strands. The first two β-strands (β_A-β_B) are connected by a variable RT-loop region (named after the arginine and threonine residue pair in the Src kinase). The β_B-β_C strands are separated by the N-Src loop (named after the neuronal isoform of Src that has an extension in this region), and the β_C and β_D strands are separated by the distal loop (because it is positioned in the SH3 domain opposite of the SH3 ligand-binding site). The fourth and fifth strands (β_D and β_E) are separated by a 3_{10} helix. The two β-sheets create a hydrophobic groove that binds specifically to peptides with polyproline helical conformation. The N- and C-termini are close to each other, which is also a general feature of modular domains and enables their host proteins to maintain their general structure (Pawson et al., 2002).

30

A
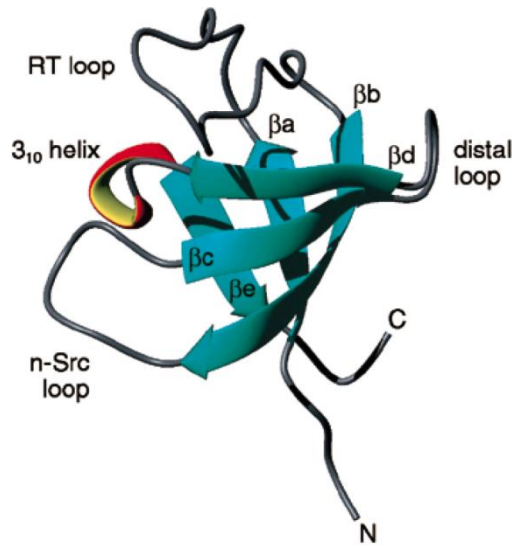
B


```
                    RT loop                N-Src loop          distal loop

      →  βA →                          → βB →              → βC →                    → βD → 3₁₀ βE →

KYVRALYDYEA-------REDDEISFKKGDIITVLEKS---------DDGWWKGRLN--------DTGREGTFPSNYVEEID    Consensus

TTFVALYDYES-------RTETDLSFKKGERLQIVNNT---------EGDWWLAHSL--------STGQTGYIPSNYVAPSD   Src
TTFVALYDYES-------RTETDLSFKKGERLQIVNNT---RKVDVREGDWWLAHSL--------STGQTGYIPSNYVAPSD   N-Src
IIVVALYDYEA-------IHHEDLSFQKGDQMVVLEES---------GEWWKARSL--------ATRKEGYIPSNYVARVD    Hck
LKGRALYDFHS-------ENKEEISIQQDEDLVIFSET---------SLDGWLQGQN--------SRGETGLFPASYVEIVR   SNX30
MEAIAKYDFKA-------TADDELSFKRGDILKVLNEE---------CDQNWYKAEL---------NGKDGFIPKNYIEMKP   Grb2(I)
LVVRAKFNFQQ-------TNEDELSFSKGDVIHVTRVE---------EGGWWEGTLN---------GRTGWFPSNYVREVK    β-PIX
CAVKALFDYKA-------QREDELTFIKSAIIQNVEKQ---------EGGWWRGDYG---------GKKQLWFPSNYVEEMV   α-PIX
MPAYVKFNYMA-------EREDELSLIKGTKVIVMEKC---------SDGWWRGSYN----------GQVGWFPSNYVTEEG   Nck1(II)
EPFQALYNYTP-------RNEDELELRESDVIDVMEKC---------DDGWFVGTSR--------RTKFFGTFPGNYVKRL    ArgBP2(III)
TPYRAMYQYRP-------QNEDELELREGDRVDVMQQC---------DDGWFVGVSR--------RTQKFGTFPGNYVAPV    Vinexin(III)
FSYQALYSYIP-------QNDDELELRDGDIVDVMEKC---------DDGWFVGTSR--------RTKQFGTFPGNYVKPLY   Ponsin(III)
SVYVAIYPYTP-------RKEDELELRKGEMFLVFERC---------QDGWFKGTSM--------HTSKIGVFPGNYVAPVT   POSH1(III)
NVYLALYAYKP-------QKSDELELHKGEMYRVLEKC---------QDGWFKGASL--------RTGVSGVFPGNYVTPVS   POSH2(III)
```

Figure 4. (**A**) Structure of the Hck SH3 domain. Reprinted from (Horita et al., 1998), with permission from Elsevier. (**B**) Aligned sequences of some SH3 domains discussed in the text. At top, the secondary structure elements and the consensus SH3 domain sequence (Larson and Davidson, 2000) are shown. The conserved residues involved in canonical peptide binding (Saksela and Permi, 2012) are highlighted in specific colors. Light blue indicates the residues that are crucial for the formation of xP pockets, and light red indicates the conserved residues that form the specificity pocket. A roman numeral after the SH3 domain indicates which of the multiple SH3 domains is in depicted (e.g. POSH2 (III) denotes the third SH3 domain of POSH2).

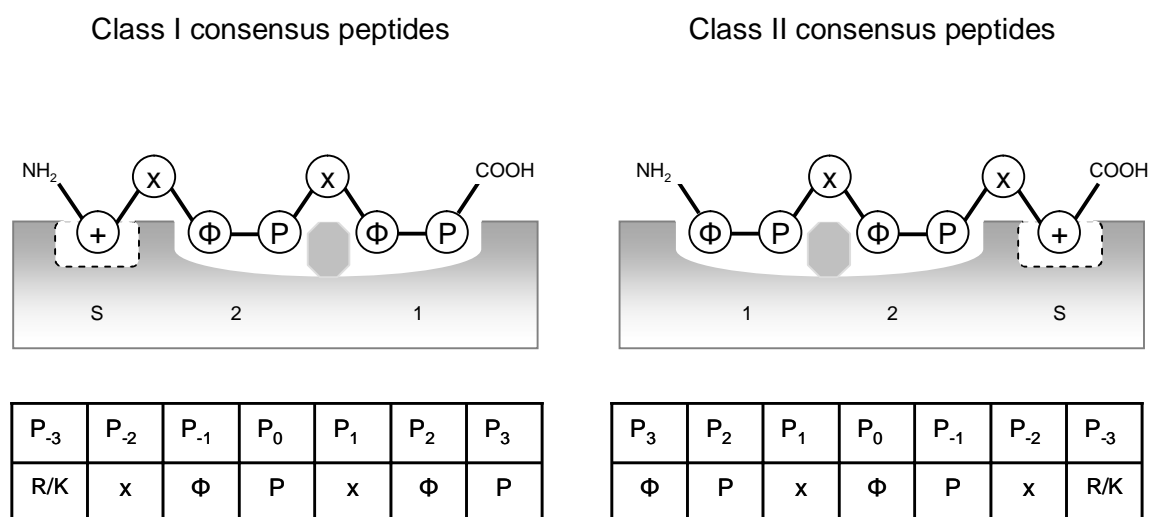## 5.4.2   SH3 / ligand binding site and specificity

The SH3 domain contains a relatively shallow ligand-binding surface that is flanked by the β_C and β_D strands, the C-terminal part of the β_B strand and N-Src loop, and the tip of the RT loop. This ligand-binding surface is formed by the side chains of highly conserved amino acids (Figure 4B) (Musacchio et al., 1992; Yu et al., 1992). The most conserved residues include the first tryptophan in the generally conserved WW dipeptide at the beginning of the β_C strand, a proline residue at the end of the β_D strand and the asparagine and tyrosine residues in the 3_{10} helix. In addition, two aromatic tyrosines at the beginning of the RT loop are especially conserved and often form the ALYDY-sequence motif. These residues form a generally hydrophobic surface, in which the side chains are stacked against each other. This hydrophobic area is wedged between the RT- and N-Src loops, whose conserved residues provide specificity for ligand binding and form the ligand-binding site (Kaneko et al., 2011; Yu et al., 1994). Non-conserved regions in the RT- and N-Src loops provide more specificity for ligand binding. Experiments utilizing phage display methods have shown that the SH3 domain binding properties could be profoundly altered by modifying the non-conserved region within the RT-loop (Hiipakka et al., 1999; Hiipakka and Saksela, 2007). Moreover, a positively charged residue (arginine or lysine) within the N-Src loop of the epidermal growth factor receptor pathway substrate 8 (Eps8) family reinforces the unique ligand-binding mode of this protein family (Aitio et al., 2008).

5.4.2.1   *Typical ligand motifs recognized by SH3 domains*

Early studies of SH3 domains indicated that it binds to proline-rich sequences (Ren et al., 1993). Subsequent structural studies on SH3/ligand complexes and phage display studies (reviewed in (Kay et al., 2000)) indicated that SH3 domains specifically bind two consensus peptides containing ΦP moieties, where Φ is usually a hydrophobic residue. Structural analysis of SH3 domain/ligand complexes (Feng et al., 1994; Lim et al., 1994; Musacchio et al., 1994; Yu et al., 1994) revealed that a typical SH3 ligand adopts a left-handed polyproline II (PPII) helical conformation. The PPII helix has three amino acids per turn and is approximately triangular in cross-section. Because of this conformation, each proline in the ΦPxΦP core motif

faces the same side of the helix, and the connecting residue (x) between ΦP moieties faces the other side of the helix (Figure 5). The presence of proline, which is quite common as a connecting residue in the core motif, has been suggested to stabilize the structure of the PPII helix (Yu et al., 1994).

Class I consensus peptides · · · · · · · · · · · · · · · Class II consensus peptides

P$_{-3}$	P$_{-2}$	P$_{-1}$	P$_0$	P$_1$	P$_2$	P$_3$
R/K	x	Φ	P	x	Φ	P

P$_3$	P$_2$	P$_1$	P$_0$	P$_{-1}$	P$_{-2}$	P$_{-3}$
Φ	P	x	Φ	P	x	R/K

Figure 5. Binding of class I and class II ligands to SH3 domains. Modified from (Aitio et al., 2008). The ligand residues are numbered according to (Lim et al., 1994). Residues of the proline-rich dipeptides (ΦP) contact the two binding pockets (indicated by numbers 1 and 2), and a positively charged amino acid (R, arginine / K, lysine) interact with the specificity pocket (S) as indicated by the dotted line. The basic residue is located at the N-terminus of the class I peptides and at the C-terminus of the class II peptides.

The structural similarity of the ligand PPII helix viewed from its C- or N-terminus allows ligands to bind in two orientations: NH$_2$ to COOH (class I; plus orientation) and COOH to NH$_2$ (class II; minus orientation). The two classes of SH3 ligands differ in the location of the basic amino acid, which determines the orientation of the ligand on the surface of the SH3 domain (Feng et al., 1994). Class I ligands share the consensus of +xΦPxΦP, and class II ligands share the consensus of ΦPxΦPx+ (where + denotes a basic amino acid). In the SH3 domain, there are two hydrophobic ligand-binding pockets that are occupied by ΦP dipeptides,

whereas the basic residue (+) flanking the ΦPxΦP core motif interacts with the third negatively charged "specificity pocket". These positively charged residues (lysine or more commonly arginine) in the class I and class II peptides interact with the same residue in the SH3 domain RT-loop (see Figure 4B); thus these residues play a key role in determining the orientation of the ligand.

Some additional features of the SH3 domain structure influence on the ligand-binding orientation. For example, tilting of conserved tryptophan indole ring (the first tryptophan residue of the conserved WW dipeptide in the β_C strand; see Figure 4B) toward or away from the proline in the ligand, influences the ligand-binding specificity whether the SH3 domain binds to class I or class II ligands (Fernandez-Ballester et al., 2004).

Some SH3 domains, such as those found in Src and phosphoinositide 3-kinase (PI3K), can bind ligands in both the plus and minus orientations (Feng et al., 1994; Yu et al., 1994). However, some SH3 domains (e.g., those found in Abl) may prefer only one orientation (Musacchio et al., 1994).

5.4.2.2   Atypical SH3-ligand interactions

Many ligands that bind to SH3 domains do not conform to the canonical class I or class II "PxxP"-motifs described above (Kaneko et al., 2008; Mayer, B.J. and Saksela, K., 2004). These include cases where the peptide does not contain the classical PxxP-consensus and cases where the peptide does not contain canonical PxxP-sequences but contains a defined structural element. Finally, complex interactions may utilize recognition strategies that differ from typical ligand recognition mechanisms (Hiipakka, M., 2005; Mayer, B.J. and Saksela, K., 2004).

Quite many SH3 domains have been reported to bind ligands that do not contain canonical PxxP-motifs. SH3 domains of Eps8 family members (Aitio et al., 2008; Mongiovi et al., 1999; Saksela and Permi, 2012) and the N-terminal SH3 domain of Nck1 (Kesti et al., 2007) recognize sequences containing a PxxDY motif. The Eps8L1 SH3 domain has a smaller hydrophobic pocket, which is not optimal for binding conventional PxxP peptides (Aitio et al., 2008). Another example comes from the SH3 domains of α- and β-PAK-interacting exchange factors (PIXs), which bind to the PAK2 atypical proline-rich sequence, PPPVIAPRP (Manser et al., 1998).

A PAK2 peptide containing this non-contiguous class II ligand-binding motif (PxxxPR) does not adopt the polyproline II helical conformation but forms a 3_{10} helix upon interaction with the β-PIX SH3 domain (Hoelz et al., 2006). Moreover, there are also SH3 domain-binding motifs that bind to the hydrophobic ligand-binding groove of the SH3 domain, but these motifs do not include prolines. For example, Fyn and Lck SH3 domains could bind to a RKxxYxxY motif in Src kinase-associated protein of 55 kDa (SKAP55) adaptor protein (Kang et al., 2000). Fyn and Lck SH3 domains are class I binders and could interact with this motif, suggesting that the recognition motif (RKxxYxxY) is similar to the class I consensus recognition sequence (+xΦPxΦP). This proline-independent recognition motif enables SKAP55 to interact with an alternative way with the SH3 domains that otherwise recognize ligands with class I proline-rich consensus motifs (Kang et al., 2000).

An example of an interaction, in which the ligand contains an atypical ligand recognition surface, comes from the studies of peroxisome associated proteins, peroxins. The *S. cerevisiae* Pex13p SH3 domain binds to an α-helical element of Pex5p (25 amino acid in length) that is sufficient and necessary for the SH3 interaction (Barnett et al., 2000). The Pex13p SH3 domain also binds Pex14p, which possesses a canonical PxxP-motif. Structural studies demonstrated distinct binding sites on opposite sites of the Pex13p SH3 domain for Pex5p and Pex14p, allowing simultaneous binding of these two ligands (Pires et al., 2003).

Complex interactions can be achieved by strategies that are distinct from the fundamental SH3-ligand recognition. Monomeric proteins may form dimers or higher order oligomers via three-dimensional (3D) domain swapping, in which proteins exchange complementary substructures. Eps8, which contains a single SH3 domain, can dimerize by swapping beta strands between two Eps8 SH3 domains (Kishan et al., 1997). Another example of SH3 domain versatility in protein complexes can be observed in membrane-associated guanylate kinase (MAGUK) proteins, whose SH3 and guanylate kinase-like (GK) domains interact with each other intra- or intermolecularly (McGee et al., 2001). In a model of MAGUK oligomerization, the core SH3 domain of one protein interacts with the subdomain between the SH3 and GK domains of another protein via 3D domain swapping to reassemble a complete SH3 fold (McGee et al., 2001).

5.4.3 Nef, ADAM15 and PAK2 as SH3-binding proteins

Nef is a small protein of approximately 27-34 kDa and is found in human and simian immunodeficiency viruses (HIV and SIV) and is important for HIV infectivity (reviewed in (Greenway et al., 2003; Renkema and Saksela, 2000)). Nef binds SH3 domains, which is important for specific cellular functions of Nef; these functions include modulating signaling pathways and enhancing HIV replication (Renkema and Saksela, 2000; Saksela, 2011). Nef interacts with different SH3-domain-containing proteins, including the Src family kinases Hck, Lyn, Fyn, Src and Lck and the guanine nucleotide exchange factor Vav (Arold et al., 1998; Renkema and Saksela, 2000). Hck and Lyn bind Nef with highest affinity, whereas Lck, Fyn and Src weakly bind to Nef (Lee et al., 1995; Saksela et al., 1995). Nef binds the Hck SH3 domain with an affinity that is particularly high for SH3-mediated binding (K_D value of 250 nM (Lee et al., 1995). However, a 12-residue long peptide overlapping the Nef PxxP motif had low affinity for Hck (K_D value of 91 µM), suggesting that additional regions in Nef are important for its ability to bind the Hck SH3 domain. Both the class II SH3-binding consensus motif in Nef as well as regions more distally from the canonical PxxP site, such as the hydrophobic pocket in Nef and the tip of the Hck RT-loop in the SH3 domain, coordinate together to result in strong and specific binding of Nef and Hck (Lee et al., 1995; Lee et al., 1996). Compared to the high affinity binding of Hck to Nef, the highly homologous SH3 domain of Fyn demonstrated strikingly weaker affinity to full-length Nef ($K_D > 20$ µM) (Lee et al., 1995). Hck is expressed in macrophages and Nef/Hck interaction results in deregulated macrophage behavior. Additionally, this interaction may contribute to the pathogenesis of acquired immunodeficiency syndrome (AIDS) (Saksela, 2011). A similar SH3 domain capable of binding Nef with high affinity (such as that observed in Hck) has not yet been found in T lymphocytes, which are the major target cells for HIV infection.

p21-activated kinases (PAKs) are pivotal signaling molecules that play important roles in remodeling the actin cytoskeleton, in apoptosis and malignant transformation (Bokoch, 2003). PAK kinases are serine/threonine kinases that are highly conserved among eukaryotes. Almost all eukaryotes encode at least one *PAK* gene, indicating a common origin and suggesting that these kinases have an important function for the cell (Hofmann et al., 2004). In higher eukaryotes, the

PAK family is divided into two subgroups. Group A PAKs (PAK1-3) act as downstream effectors for the small p21-GTPases, Rac1 and Cdc42 (Arias-Romero and Chernoff, 2008). Group A PAKs have similar structures and they contain an N-terminal regulatory domain and a C-terminal kinase domain (Figure 6). PAKs typically have Pro-rich motifs within their N-terminus, and these motifs act as docking sites for SH3 domains. PAKs have been reported to bind to different SH3-domain-containing proteins, including Grb2 (Puto et al., 2003), Nck (Bokoch et al., 1996; Galisteo et al., 1996), PIX (Manser et al., 1998) and Arginine-binding protein 2 (ArgBP2) (Yuan et al., 2005). The binding of PAK1 to Grb2 and to Nck has been suggested to be important in growth factor signaling (Bokoch et al., 1996; Galisteo et al., 1996; Puto et al., 2003). The PIX SH3 domain binds to PAK with an affinity that is higher than is normally observed for SH3 domains (K_D value of 24 nM) (Manser et al., 1998). An arginine residue in the PAK2 core SH3-binding motif (PxxxPR) coordinates with additional residues that are located C-terminal to the core SH3-binding site, to enable additional interactions with the RT-loop of the PIX SH3 domain. A sum of these interactions mediates the high-affinity binding to PIX (Hoelz et al., 2006; Saksela and Permi, 2012).

PAKs also contain a binding site for p21-GTPases, which is called the Cdc42 and Rac interactive binding domain (CRIB); this domain forms the binding site for these small GTPases. PAK is commonly activated by interacting with GTP-loaded Cdc42 or Rac1. Following the binding of these GTPases, PAKs become serine/threonine-autophosphorylated and highly activated (Figure 7A) (King et al., 2000). Tyrosine phosphorylation of PAKs has also been reported in several studies. PAK2 activity induced by p21-GTPases was strongly potentiated by tyrosine phosphorylation resulting in superactivated PAK2 (Renkema et al., 2002). Moreover, Src family kinase-mediated tyrosine phosphorylation of PAK2 was shown to be dependent on conformational changes induced by p21-GTPases (Renkema et al., 2002). PAK2 becomes tyrosine phosphorylated only in its N terminus and of the three N-terminal tyrosines (Y130, Y139 and Y194), Y130 is the major phosphoacceptor site, and Y194 is an additional putative tyrosine phosphorylation site (Renkema et al., 2002).
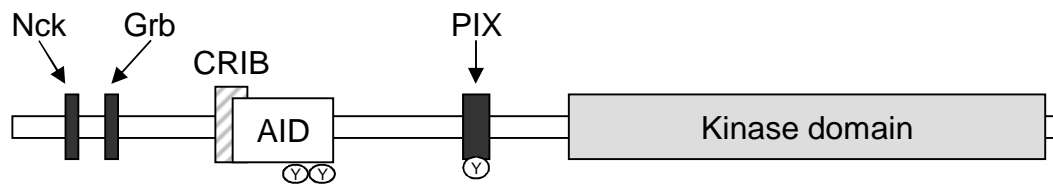
Figure 6. Schematic diagram of the PAK2 structure. The striped box indicates the CRIB (Cdc42 and Rac interactive binding domain). Dark boxes indicate the Pro-rich motifs and the specific SH3 domain-containing proteins that interact with these sites are noted. The kinase domain is depicted in light grey, and the autoinhibitory domain (AID) is shown in white. Small circles represent the N-terminal tyrosines, Y130, Y139 and Y194. When these tyrosines are phosphorylated, they may serve as potential SH2 domain docking sites.

The exact mechanism of PAK2 superactivation is not known, and this raises the possibility that the phosphotyrosine residue may serve as an interaction site for activating molecules such as SH2-domain-containing proteins. Active PAK kinases have been reported to induce the formation of lamellipodia, membrane ruffling and filopodia (Sells et al., 1997), which are characteristic cytoskeletal alterations in motile cells. PAKs have been implicated in the control of normal cellular processes, and their overexpression and/or superactivation has been observed in cancer progression (Bokoch, 2003; Kumar et al., 2006; Molli et al., 2009). Elevated PAK activities have been described in human breast cancer cell lines (Mira et al., 2000), and PAK kinase activity correlates with invasiveness of breast cancer cells and breast tumor grades (Vadlamudi et al., 2000).

A disintegrin and a metalloproteases (ADAMs) comprise a family of proteases that are associated with the proteolytic processing of cell surface protein ectodomains (White, 2003). In addition to influencing cell-cell interactions by shedding ligands, they are engaged in intracellular signaling. The identification of several cytosolic ligands has suggested that ADAM15 is important in cellular signaling. The cytosolic portion of ADAMs, such that of ADAM15, contains proline-rich regions that can bind to many SH3 domain-containing proteins such as Src family kinases (Shimizu et al., 2003) and the multidomain protein Fish (five SH3 domains, also called Tks5) (Abram et al., 2003). The ADAM15 cytoplasmic tail also binds other SH3-domain-containing proteins, including the following

proteins involved in endocytosis and cell sorting: endophilin-1, sortin nexin 9 (SNX9) and pacsin 3 (Howard et al., 1999).
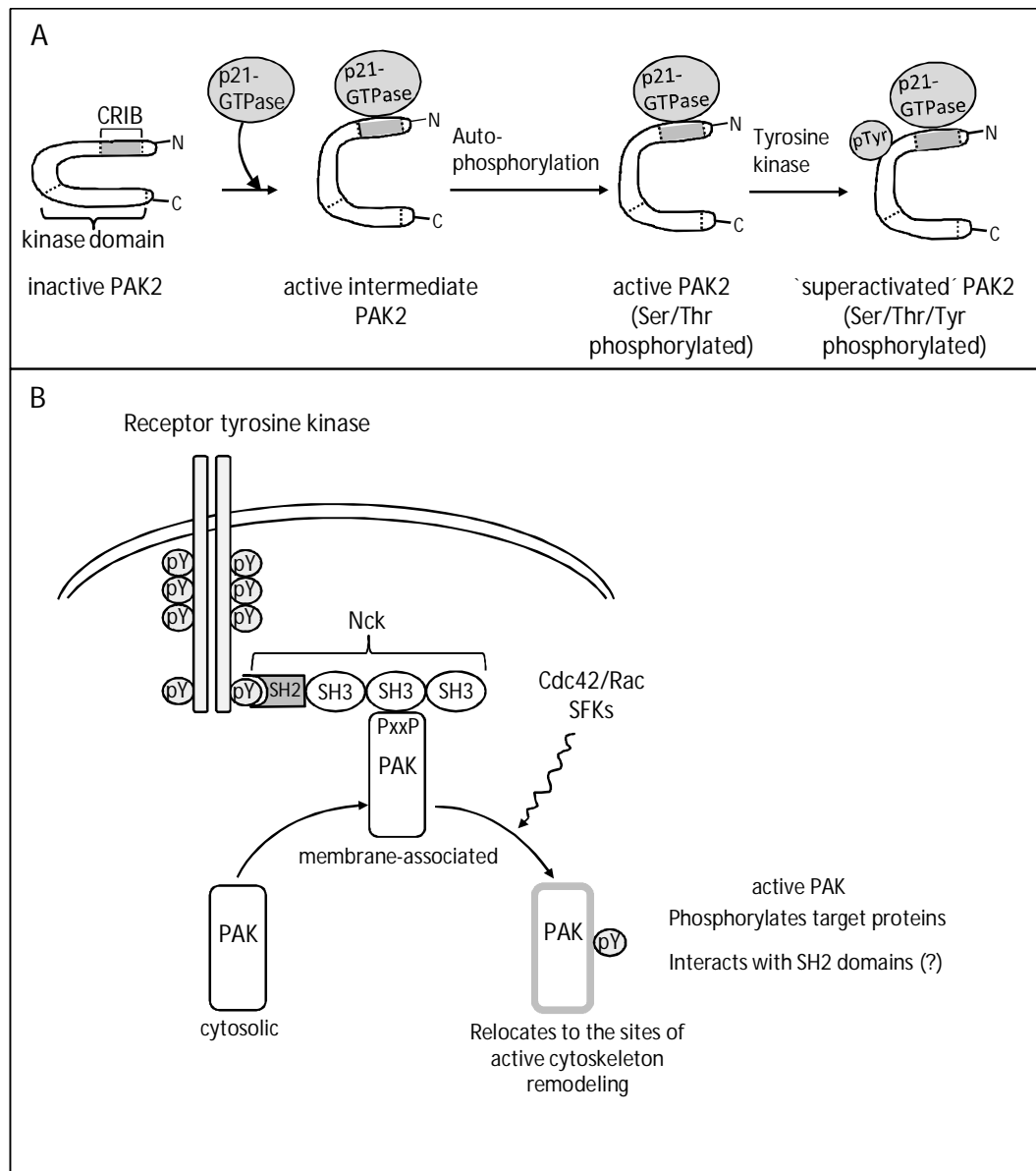
Figure 7. (**A**) Simplified model of PAK2 activation. (**B**) Schematic model of PAK kinase interactions, which are dynamically regulated within a cell. Here, inactive PAK is translocated from the cytosol to the plasma membrane by Nck via SH3/PxxP-interactions. (Nck interacts with Tyr-phosphorylated receptors). In the plasma membrane, PAK can be activated by small GTPases and further by non-receptor Src family kinases (SFKs). Active PAK may interact via its pTyr residues with SH2 domains and PAK can phosphorylate target proteins via its active kinase domain.

5.4.4 Predicting SH3 domain interactions

The human proteome contains hundreds of SH3 domains and SH3 target molecules, which in theory generate a large number of potential SH3 interactions. To determine which of these SH3/ligand interactions occur and are biologically relevant, it helps us to understand how signaling networks regulate normal cellular behavior and how they are deregulated in many diseases. Localization of the proteins and the local concentration of their binding partners are important factors for determining whether the SH3/ligand interactions occur *in vivo*. Nevertheless, *in vitro* SH3/ligand interactions with high specificity and affinity may also be physiologically meaningful. This has prompted the development of tools to predict potential SH3/ligand pairs (reviewed in (Hiipakka, M., 2005; Mayer, B.J. and Saksela, K., 2004)).

Several studies have been conducted to predict potential SH3/ligand pairs using computational strategies based on structures of the SH3/ligand complex and the SH3 binding preferences of target peptides (Brannetti et al., 2000; Panni et al., 2002). The results from these types of studies provide only a model of the interaction, and these studies are limited that a selective or strong SH3/ligand interaction often involves unique molecular contacts that are not sufficiently taken into account by homology modeling. To overcome these limitations, Tong and colleagues combined computational and experimental approaches to predict SH3 interaction networks in *S. cerevisiae* (Tong et al., 2002). They used twenty different *S. cerevisiae* SH3 domains to screen a random peptide library via phage display, and they established the consensus sequence for each SH3 ligand. Based on these results, the yeast proteome was searched for potential natural SH3 ligands, which were used to generate a protein-protein interaction network via computation methods. Interactions that were predicted by the phage-display screen were then assessed by using the yeast two-hybrid system, and the intersection of the experimental and predicted networks was determined. Most of the interactions that were identified by the predictive and experimental data sets could be verified by co-immunoprecipitation assays *in vivo*, suggesting that this method could be useful for identifying biologically meaningful interactions.

Landgraf and colleagues introduced the WISE (whole interactome scanning experiment) approach, which was better equipped for meeting the demands of

40

screening a more complex human proteome (Landgraf et al., 2004). They extended the number of most frequent ligands for eight yeast SH3 domains by identifying "relaxed consensus" sequences for the ligands instead of using "strict consensus" sequences in the yeast proteome screening (Tong et al., 2002). They synthesized approximately 1500 peptides on membranes and measured the binding of these peptides to each SH3 domain. Some peptides had overlapping binding specificities to the target SH3 domains, whereas other peptides were more specific. Overall, there was a decent correlation between predicted and observed interactions. This approach was then used to analyze the human proteome to identify target proteins for the amphiphysin-1 and endophilin-1 SH3 domains. Of the 3774 peptides assessed, the peptides derived from the target proteins of these SH3 domains were among the best binders, suggesting that these identified interactions were relevant. More recently, the WISE technique was utilized to identify interactions between 15 human SH3 domains and the proline-rich motifs of two oncogenic viruses (Carducci et al., 2010). Fourty-six viral proline-rich peptides were synthesized on membranes, and the binding of these peptides to various SH3 domains was measured. This approach identified 114 new putative SH3/proline-rich ligand interactions and confirmed that the Hck and Fyn SH3 domains bind to viral protein ligands as was previously described in the literature.

Xu and colleagues introduced recently a new proteome-wide approach for identifying Abl1 SH3 binding peptides (Xu et al., 2012). They integrated computational strategies based on SH3/ligand structures, interactions and conservation during evolution, and SH3/ligand-binding preferences, and a peptide microarray technology. They used a virtual mutagenesis method to determine the ligand-binding profile of the Abl1 SH3 domain. Each residue in the peptide was mutated systematically to the other natural amino acids, and the SH3/ligand binding profile that was generated was then used as a scoring matrix to search approximately 69 000 PxxP-motif-containing peptide sequences in the human proteome. The conservation of the Abl1 SH3/binding partners was assessed across species, and the peptides in the Abl1 SH3/peptide complex model were analyzed computationally. Approximately 700 peptides were tested by peptide microarray analysis on the basis of the most confident peptide partners of Abl1 SH3 domain, and 237 peptides from putative protein binders were identified. Though they identified several putative interacting partners of Abl1, they only found approximately 25% of the previously

described Abl1 SH3-binding partners, reflecting the challenge of a proteome-wide approach for identifying binding partners.

Even though it is evident that the SH3/ligand screens provide us information regarding both known and novel interactions, it appears that these screens are more adept at identifying possible interactions instead of revealing the more likely ones. SH3 domains are thought to bind to ligands with low specificity and affinity, typically with K_D values in the micromolar range. However, this aspect is mainly based on the data using short linear peptides as SH3 ligands, and these peptides exclude the SH3-binding regions outside of the core binding motif. Nonetheless, several examples of SH3 interactions have been reported with binding affinities in the nanomolar range (Berry et al., 2002; Lee et al., 1995; Manser et al., 1998). Computational approaches have limitations in recognizing SH3/ligand patterns. These methods also have difficulty taking into account for example the subcellular localization of a protein and the local protein concentrations that are often mediated by other protein-protein interactions and the natural context, which are all important for protein interactions *in vivo*.

5.5 POSH proteins and POSH-mediated cellular functions

POSH (plenty of SH3 domains) proteins are a relatively new group of multidomain scaffold proteins, and two members of this family have been previously described in the literature. Hereafter, we will refer to the original POSH protein identified by Tapon and colleagues (Tapon et al., 1998) as POSH1. The more recently identified POSH protein was discovered by Wilhelm and colleagues and named POSH2 (and recently re-named by the same authors as POSH-eliminating RING protein (POSHER)); this protein will be denoted as POSH3 in this doctoral thesis (Wilhelm et al., 2007a; Wilhelm et al., 2012). This change in nomenclature is indicated in Figure 9 and will also be discussed in more detail in *Results*.

POSH proteins are involved in modulation of signaling cascades, leading to cytoskeletal reorganization (Taylor et al., 2008) and the regulation of programmed cell death, apoptosis (Kukekov et al., 2006; Lyons et al., 2007; Wilhelm et al., 2007a; Wilhelm et al., 2012; Xu et al., 2003; Xu et al., 2006). POSHs are

multidomain proteins that function as scaffolds, particularly for c-Jun N-terminal kinase (JNK; or stress-activated protein kinase (SAPK)) signaling pathways. POSH-mediated JNK signaling has also been studied in neuronal development and in neuronal apoptosis (Kim et al., 2005; Taylor et al., 2008; Wilhelm et al., 2007b; Zhang et al., 2005; Zhang et al., 2006; Zhang et al., 2009). POSHs can regulate the post-translational modification of proteins by acting as an ubiquitin E3 ligase, which promotes the attachment of ubiquitin to target proteins (Xu et al., 2003; Wilhelm et al., 2007a). POSH proteins can regulate the turn-over of cellular target proteins via its E3 ligase activity, and this function has been implicated in other POSH-mediated cellular processes, including cell homeostasis (Keil et al., 2010; Lin et al., 2009; Tuvia et al., 2007) and virus release (Votteler et al., 2009). The central functions of POSH proteins and the cellular phenomena in which they function will be discussed in further detail below, and their roles in JNK signaling and protein ubiquitination will be emphasized.

5.5.1   JNK signaling and cell death

The highly conserved mitogen-activated protein kinase (MAPK) signal transduction pathways allow cells to respond to various extracellular stimuli. Mammals contain three well-characterized MAPK subfamilies: extracellular signal-related kinases (ERKs), JNKs and p38 MAPKs (Chang and Karin, 2001; Plotnikov et al., 2011). MAP kinases connect extracellular stimuli to regulatory targets in the cell, therefore controlling the cellular response to its environment. These responses control many cellular functions, including proliferation, differentiation, development, inflammatory responses and apoptosis. The MAP kinases form a protein kinase activation system of three levels; MAPK is phosphorylated and activated by MAP kinase-kinase (MAPKK), which in turn is phosphorylated and activated by MAP kinase-kinase-kinase (MAPKKK) (Davis, 2000; Plotnikov et al., 2011).

JNK signaling can mediate opposing cell fates, including apoptosis, cell proliferation and cell survival. The outcome of the activated JNK signaling depends on the prior stimuli, cell type, the activity of other signaling pathways and the duration of JNK activation (Davis, 2000; Weston and Davis, 2007). Sustained JNK

activity is required for apoptotic signaling, whereas transient JNK activation is associated with cell survival (Ventura et al., 2006).

The JNK signaling pathway is primarily activated in response to cellular stress and cytokines, which trigger the activation of the pathway in which many MAPKKKs, such as MAPK/ERK kinase 1-4 (MEKK1-4), mixed lineage kinase 1-4 (MLK1-4), dual leucine zipper-bearing kinase (DLK), and apoptosis signal-regulating kinase 1 (ASK1), have been found to phosphorylate and activate the MAPKK MKK4 (also called SAPK/Erk kinase 1 (SEK1)) and MKK7, which in turn synergistically phosphorylate and activate JNK (Dhanasekaran and Reddy, 2008; Weston and Davis, 2002). Once activated, JNKs (three family members, JNK1-3) can promote apoptosis via two different mechanisms. Firstly, activated JNK can translocate into the nucleus and control gene expression by regulating the activity of transcription factors, such as c-Jun, which subsequently leads to increased expression of pro-apoptotic genes (Davis, 2000; Weston and Davis, 2007). Secondly, in response to different stimuli, such as DNA damage, activated JNK can translocate to mitochondria where it regulates the activity of anti-apoptotic proteins, and eventually leading to the release of cytochrome c and the activation of caspase signaling (Dhanasekaran and Reddy, 2008) (as illustrated in Figure 8).

The most classical nuclear substrate for JNK is c-Jun. Phosphorylation of c-Jun by JNK leads to the formation of activating protein 1 (AP-1) and increases its transcriptional activity (Karin, 1995). Many genes have binding sites for the AP-1 complex in their promoter region, some of which encode for the proteins involved in apoptosis. AP-1 is a dimeric transcription factor complex and c-Jun is a major component of this complex (Bohmann et al., 1987).
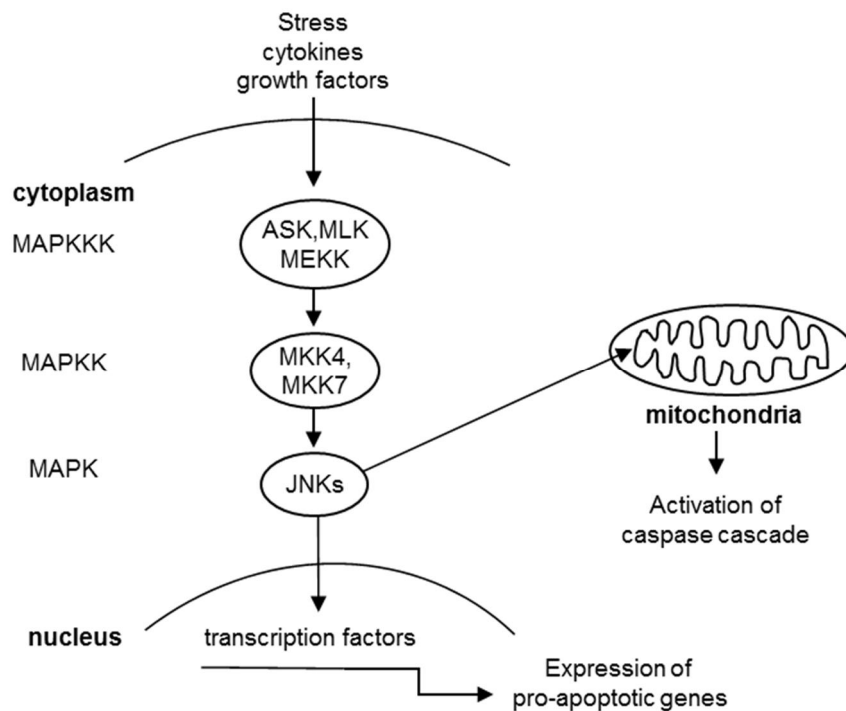
Figure 8. Schematic overview of the JNK MAPK signaling cascade in apoptosis. The MAPK module contains a MAPKKK, which phosphorylates MAPKK. MAPKK then phosphorylates and activates MAPK (c-Jun N-terminal kinase, JNK). JNK activation is followed by nuclear translocation and phosphorylation of transcription factors in the nucleus, and these factors further control the cellular response (apoptosis). JNK activation can also promote apoptosis via mitochondrial translocation and promoting activation of the caspase cascade.

5.5.1.1 Scaffold proteins in the JNK signaling cascade

Scaffold proteins function as molecular platforms that enable the assembly of multiprotein signaling complexes. These scaffold proteins bind and recruit signaling molecules into close proximity, facilitating rapid flow of information between signaling partners. Scaffold proteins provide specificity to the signal transduction and regulate cellular signaling by promoting desired signaling outcomes while limiting inappropriate signaling cascade cross-talk. Single eukaryotic cells typically contain multiple MAPK pathways that have unique or shared kinases. Scaffold proteins can form separate multienzyme complexes and ensure the preferred

45

signaling fidelity, particularly when the same protein kinase functions in multiple pathways. Scaffold proteins are thought to mediate cross-talk between bound kinases, and they connect these kinases to receptors, G proteins or other signaling molecules within the cell (Dhanasekaran et al., 2007; Engstrom et al., 2010). Changes in location, composition or the amount of the specific molecules in the pathway over time are also crucial factors for the signal transduction pathways regulated by scaffold proteins (Dhanasekaran et al., 2007; Engstrom et al., 2010; Scott and Pawson, 2009).

Ste5p was the first MAPK scaffold protein to be described, and it was discovered in the budding yeast *S. cerevisiae* (reviewed in (Elion, 2001)). To date, several distinct scaffold proteins involved in the JNK signaling cascade have been identified in mammals; these proteins include filamin (also known as actin-binding protein of 280 kDa size, ABP280) (Nakagawa et al., 2010), β-arrestin 2 (McDonald et al., 2000), JNK-interactive proteins (JIPs) (reviewed in (Engstrom et al., 2010)) and POSH proteins (Wilhelm et al., 2007a; Xu et al., 2003).

5.5.2 POSH proteins in the JNK signaling cascade

POSH1 was identified as a novel Rac-binding protein and due to its multidomain structure and its ability to induce JNK and NF-κB signaling, it was proposed that POSH1 functions as a scaffold protein that facilitates the formation of a signaling complex (Tapon et al., 1998). Subsequently, studies suggested that POSH1 interacts directly with the MAPKKK MLKs (MLK1-3 and DLK) (Xu et al., 2003) and that POSH1 forms a complex with the MAPKKs MKK4 and MKK7 (Xu et al., 2003; Zhang et al., 2005) and the MAPKs JNK1 and JNK2 (Xu et al., 2003). POSH1 also forms a heterodimeric complex with JIP, another scaffold protein (Kukekov et al., 2006). POSH3 is a POSH1 homolog and maintains a quite similar domain structure. POSH3 scaffold protein also promotes JNK activation and cell death (Wilhelm et al., 2007a).

Human POSH proteins share similar structural features, which include 1) an N-terminal RING finger domain that can confer E3 ubiquitin ligase activity to the protein, 2) SH3 domains (four in POSH1 or three in POSH3) that interact with proteins containing Pro-rich motifs and 3) a unique Rac-binding site that is located

between the second and third SH3 domains in the protein sequence (Tapon et al., 1998; Wilhelm et al., 2007a) (depicted in Figure 9). POSH1 was originally identified in a yeast-two hybrid screen as a novel protein that interacts with a Rac GTPase (Tapon et al., 1998). Rac was found to interact with a unique Rac-binding site (aa 292-362 in POSH1) that did not contain a typical CRIB domain (Tapon et al., 1998), such as the domain present in the PAK2 kinase. POSH1 specifically binds Rac but not other p21 GTPases, such as Cdc42 or Rho (Tapon et al., 1998; Wennerberg et al., 2002). The Rac/POSH1 interaction has been shown to promote apoptosis (Lyons et al., 2007).
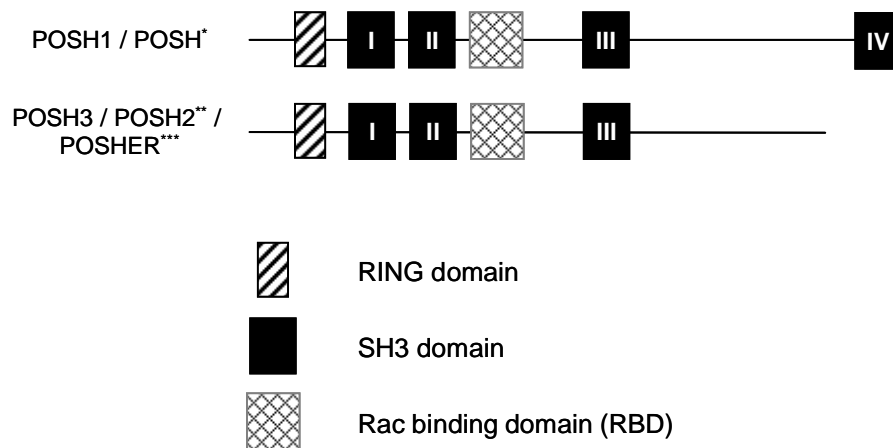
Figure 9. POSH domain structures. Representative structures of vertebrate POSH proteins are shown. (Different POSH protein nomenclature is indicated by asterisks: *(Tapon et al., 1998), **(Wilhelm et al., 2007a) and *** (Wilhelm et al., 2012)).

POSH proteins are ubiquitously expressed in different tissues. Some tissues express both POSH1 and POSH3 mRNAs, while some preferentially express one or the other gene. POSH1 transcriptional analysis revealed that while it is ubiquitously expressed in various tissues, it is most abundantly expressed in the brain, kidney, lung and liver (Tapon et al., 1998; Tsuda et al., 2010). POSH3 is most abundantly expressed in the kidney, thyroid, stomach and eye, but it also is expressed in the brain (Wilhelm et al., 2007a). POSH1 has been reported in different cellular compartments, including in trans-Golgi network and in early endosomes (Alroy et al., 2005; Kim et al., 2006).

5.5.2.1 POSH signalosome in JNK signaling

Activation of the JNK pathway is often observed in cells that are undergoing apoptosis. Rac1 acts as an upstream activator of the JNK signaling pathway (Teramoto et al., 1996). Upon activation, Rac1 becomes GTP-loaded and undergoes a conformational change, enabling Rac1 to interact with downstream effectors, such as POSH1 and MLK3 (Kukekov et al., 2006; Tapon et al., 1998; Xu et al., 2003). Binding of activated Rac1 to the MLK3 CRIB motif activates MLK3 by relieving SH3-mediated autoinhibition, which further promotes the dimerization and autophosphorylation of MLK3 (Leung and Lassam, 1998). MLK3 has been shown to bind both the N- and C-termini of POSH1, suggesting that POSH1 could simultaneously bind multiple MLKs, bringing them into close proximity and facilitating their autophosphorylation (Xu et al., 2003). Further studies demonstrated the scaffolding function of POSH1 and its ability to associate with MKK4/7 and JNK (Xu et al., 2003; Zhang et al., 2005). However, this interaction was subsequently found to be indirect and occur via JIPs, which are POSH-binding scaffold proteins (Kukekov et al., 2006).

POSH and JIPs have been shown to play a crucial role in JNK-mediated cell death. Overexpression of POSH1 promotes cell death, which is consistent with the finding that POSH1 knockdown could promote cell survival (Tapon et al., 1998; Xu et al., 2003; Zhang et al., 2005). Interestingly, neurons isolated from JIP-1 null mice were unable to induce JNK-activation or undergo apoptosis following cellular stress (Whitmarsh et al., 2001). The finding that POSH could directly interact with JIPs, suggests that a POSH-JIP apoptotic complex (PJAC) could facilitate the sequential activation of JNK pathway components (Kukekov et al., 2006) (as depicted in Figure 10).

JNK pathway components need to be regulated following apoptotic stimuli to prevent inappropriate cell death. When cell death is necessary, rapid activation of the pathway is crucial. An apoptotic stimulus in neuronal cells enhances the rapid and sustained association of POSH1, Rac1 and MLK3, suggesting that the POSH-mediated JNK-signaling complex forms after the death stimulus (Zhang et al., 2005; Zhang et al., 2009). The PJAC complex is regulated by intricate feed-forward and feedback mechanisms. Firstly, apoptotic stimuli have been demonstrated to increase the protein levels of PJAC components, indicating enhanced stability of the JNK-

pathway proteins (Xu et al., 2005; Zhang et al., 2005). Secondly, JNK pathway PJAC components are also capable of regulating one another's stability, in which the activity and phosphorylation status of the proteins play an important role. For example, inactive and dominant-negative MKK4/7, JNK and dominant-negative c-Jun were found to reduce the stability of MLKs (Wang et al., 2010; Xu et al., 2005). Moreover, POSH1 phosphorylation (on serine 127) by MLK2 stabilizes the POSH1 protein (Wang et al., 2010). Conversely, POSH1 E3-ligase activity has been shown to regulate its own turnover in cells (Xu et al., 2005).
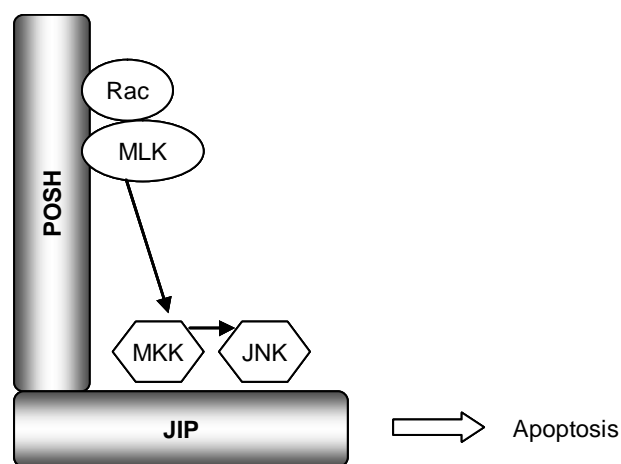
Figure 10. POSH-JIP apoptotic complex (PJAC). Modified from (Lyons et al., 2007).

The cellular decision to undergo apoptosis is determined by the balance of survival and death signals. The Akt kinases (also called protein kinase B; PKB) play a crucial role in cell survival pathways and can prevent apoptosis that is induced by a variety of death signals (Cantley, 2002). Akt serine/threonine kinases have been shown to negatively regulate the JNK-signaling cascade directly interacting with and by phosphorylating key members of the JNK-signaling complex. Rac1 is phosphorylated by Akt (on serine 71), which suppresses Rac1 activity by blocking its ability to bind GTP (Kwon et al., 2000) and decreases its interaction with MLK3 (Zhang et al., 2006). Akt phosphorylation of POSH1 (on serine 304) decreases its interaction with Rac1 (Lyons et al., 2007). Moreover, phosphorylation of MLK3 and MKK4 by Akt decreases kinase activity of these proteins and inhibits JNK

activation (Barthwal et al., 2003; Park et al., 2002). In addition to regulating of the PJAC complex via phosphorylation, Akt can regulate the PJAC by nonenzymatic mechanisms. Akt2 can directly bind to POSH1 and this interaction likely prevents MLK3 binding and the assembly of the PJAC (Figueroa et al., 2003). Another Akt isoform, Akt1, binds to JIP-1 and this interaction limits the activation of JNK pathway (Kim et al., 2002). Furthermore, JNK phosphorylation of JIP-1 led to the dissociation of Akt1 from JIP-1 (Song and Lee, 2005), indicating that there is some cross-talk between the JNK and Akt pathways.

5.5.3 POSH proteins and ubiquitination

Protein posttranslational modification is an important mechanism for regulating protein function, activity and localization. After translation, specific amino acids can be modified by the addition of functional groups, including phosphates, acetyl groups, sugar moieties, lipids and even small proteins. Ubiquitin is an example of one of these small proteins. Ubiquitination (or ubiquitylation) refers to the attachment of ubiquitin (76-amino acid protein) to a substrate protein. Ubiquitination plays a role in many cellular processes, such as signal transduction, transcriptional regulation, development and apoptosis. Moreover, ubiquitination is a mechanism for regulating the degradation of cellular proteins. Three enzymes are required for the covalent attachment of ubiquitin to the substrate in a multi-step process. These enzymes are: E1, the ubiquitin-activating enzyme; E2, the ubiquitin-conjugating enzyme; and E3, the ubiquitin-ligating enzyme. In this multi-step process, E1 activates the ubiquitin molecule in an ATP-dependent manner, and this activated ubiquitin is transferred to E2. E3 has two roles; it first specifically recognizes the substrate and simultaneously promotes the transfer of ubiquitin from E2 to the specific target protein. Additional ubiquitin molecules can be added to the first ubiquitin molecule, forming a polyubiquitinated protein (Weissman, 2001).

E3 enzymes are classified into two groups based on whether they include the HECT (homologous of E6-AP carboxyl terminus) domain or the RING (Really Interesting New Gene) finger domain. The HECT domain contains approximately 350 amino acids and has a C-terminal cysteine, which forms a covalent thiol-ester bond with ubiquitin and transfers ubiquitin from itself to the substrate molecule. The

RING finger domain is a small zinc binding domain (40-60 amino acids long), which contains a Cys3HisCys4 motif (Freemont, 2000). Most of the RING domain proteins have been shown to function as E3 ligases, which includes POSH1. RING domain-containing E3 ligases do not form an intervening intermediate with ubiquitin, but they are thought to catalyze the direct transfer of ubiquitin from ubiquitin-loaded E2 to the substrate (Deshaies and Joazeiro, 2009). E3 ligases can catalyze both mono- and polyubiquitination reactions, and ubiquitin chains can be attached via different ubiquitin lysine residues. Generally, monoubiquitination is thought to mediate the localization of the substrate protein, whereas multi-ubiquitin chains are believed to target proteins for proteasomal degradation. In the absence of external substrates, E3s can catalyze self-ubiquitination reactions, leading to their own degradation. Self-ubiquitination suggests an autoregulatory mechanism for the regulation of E3 ligase levels in cells (Xu et al., 2003).

POSH-mediated ubiquitination regulates the turnover of JNK-signaling complex proteins (Xu et al., 2005) and negatively regulates the interaction between POSH and JNK signaling complex members (Kim et al., 2006). However, POSH E3 ligase activity has also been implicated in other cellular processes. For example, POSH E3 ligase activity regulates the sorting of the virus particles to the plasma membrane and promotes virus budding (Alroy et al., 2005; Tsuda et al., 2006; Votteler et al., 2009). Moreover, POSH ubiquitination activity has been shown to regulate cell homeostasis. POSH1 E3 ligase activity downregulates potassium outflow from cells by increasing the endocytosis of potassium channels on the plasma membrane (Lin et al., 2009).

6. AIMS OF THE STUDY

Phosphorylation by tyrosine kinases is important for many critical cellular processes. Phosphorylated tyrosines form binding sites for proteins containing SH2 domains; thus we reasoned that a panel of SH2 domains could be used to probe for differences in tyrosine phosphorylation of various proteins. Therefore, we proposed to develop a high-throughput approach to profile the global tyrosine phosphorylation state of the cell. PAK2 kinases play an important role in signaling cascades. Moreover, PAK2 kinases were previously shown by our group to become tyrosine-phosphorylated leading to a highly activated kinase. For that reason, it was of our interest to find SH2-binding partners for PAK2.

SH3 domains were thought to bind their ligands with low affinity and specificity. However, we reasoned that the superior binding affinities of SH3/ligand pairs would not have evolved by chance and not be relevant. Therefore, we proposed to develop a system to identify optimal SH3/ligand pairs without the limitations associated with the use of short linear peptides or those associated with indirect approaches for protein interaction screening. Our goal was to characterize the preferred SH3 domains that bind PAK2, Nef and ADAM15 and to study the functions of the selected PAK2-ligands identified in the phage display screen.

The more detailed aims were:

1. to identify novel SH2 interactions for PAK2 and to establish assays to characterize changes in tyrosine phosphorylation in cells via use of SH2 domains

2. to devise a system for comprehensively mapping preferred SH3-mediated interactions in human proteins and to identify SH3 domains that bind PAK2, Nef and ADAM15 with high affinity

3. to characterize POSH2, a novel PAK-interaction partner, and to study its function

7. MATERIALS AND METHODS

7.1   Plasmid constructs

7.1.1   Eukaryotic expression vectors (III)

Full-length POSH2 cDNA was cloned into the pEBB vector containing the Flag epitope tag. Two sequences were used to make this construct: cDNA clone FLJ00204 containing the 3' region of POSH2 (obtained from the Kazusa DNA Research Institute) and the 5' region of human POSH2, which was PCR amplified from HeLa cell cDNA (provided by Hans Spelbrink, University of Tampere, Finland). The primers used for cloning were designed based on the predicted sequences found in several databases (NCBI Genbank reference sequence: NM_001099289). The POSH2 (amino acids, (aa) 1-70) and POSH2 (aa 46-70) fragments were cloned into the pEGFP-N1 vector (Clontech) upstream of GFP. Natural Kozak sequences were used, and the forward primers included 18 nucleotides upstream of ATG. Full-length POSH1 (NCBI Genbank reference sequence: NM_020870) and POSH2 were cloned into the pEBB vector containing an N-terminal Flag-tag. The RING domain mutations (CH-AA; POSH1 C28A/H30A and POSH2 C73A/H75A) and the CRIB domain mutant (S404P) were made via overlapping PCR using mutagenic primers.

The 3xAP-1-luc (luciferase) reporter construct (containing three binding sites for AP-1) and the pLacZ plasmid, created by inserting β-galactosidase cDNA into the pEBB vector were both provided by Kalle Saksela at the University of Helsinki, Finland. The pMT-123-Ubi (ubiquitin)-HA (Ubi-HA) plasmid was a gift from Daniela Ungureanu at the University of Tampere, Finland.

7.1.2 Bacterial vectors (I-III)

The GST (Gluthathione S-transferase)-SH3 domain fusions used in study **II** were generated by transferring the SH3 fragments from the pCR-Script clones into the pGEX-4T-1 vector (Amersham Biosciences). The GST-Nef encoding vector has been previously described (Hiipakka et al., 1999; Manninen et al., 1998). The wild-type N-terminal regulatory domain of PAK2 (PAK2N), containing residues 1-212, was subcloned into the pGEX-4T-1 vector. The POSH1 minimal Rac-binding site (aa 292-362) (Tapon et al., 1998) and the same region in POSH2 (aa 369-439) were cloned into the pGEX-4T-1 vector (**III**). POSH2 CRIB domain mutations (ISP, [I403N,S404P,P406A], I403N, S404P and P414A) and control mutants (L385A and P414A) were constructed by overlapping PCR. Rac1 and Cdc42 were cloned into the pGEX-PP vector, which contains an N-terminal biotin purification tag (BPT) (Heikkinen et al., 2008).

Table 1. The PAK2, POSH and POSH2 mutations used in this study.

Mutant	Acronym	Reported functional consequences	Reference
PAK2			
Y130F Y139F Y194F Y130F,Y139F,Y194F	-	Disrupted phosphorylation of tyrosine residues	(Renkema et al., 2002)
POSH1			
C28A,H30A	CH-AA	RING finger mutation	(Kim et al., 2005)
POSH2			
C73A,H75A	CH-AA	RING finger mutation	
I403N S404P I403N,S404P,P406A	-	p21-GTPase binding deficient	(Zhao et al., 1998)

cDNA clones for the human SH2 domains (Liu et al., 2006) are described in greater detail in the original publication (**I**). SH2 domains were cloned into the pGEX fusion vectors (Amersham Biosciences).

PAK2N was cloned into the pET23d vector (Novagen) for bacterial expression of recombinant His-tagged protein. The mutant PAK2N (Y130F, Y139F, Y194F and

[Y130F,Y139F,Y194F]) sequences have been described previously (Renkema et al., 2002), and these sequences were subcloned into the pET23d vector using PCR.

Maltose binding protein (MBP)-fusion constructs were generated by cloning relevant ADAM15 and Nef sequences into the pMAL-c2 vector (New England Biolabs).

All of the constructs used in this study were confirmed by sequencing.

7.1.3   Phagemids (II)

To produce multivalent phagemids for SH3 expression, GeneOptimizer^{TM} software (GENEART GmbH) was used to design DNA coding sequences to create a synthetic library of human SH3 domains. These genes were generated by assembling oligonucleotides in a single-step PCR-based method and they were cloned into the pCR-Script vector (Stratagene). The correct SH3 clones were cloned into the vector pG8JH, which is a derivative of pG8H6 and is used for multivalent phage-display (Jacobsson and Frykberg, 1996).

7.2   Antibodies (I-III)

The following primary antibodies were purchased: mouse anti-c-Myc (Roche), mouse anti-Flag (Sigma), mouse anti-HA (Nordic BioSite AB), mouse anti-GFP (Zymed), goat anti-GST (Amersham Biosciences) and rabbit anti-FAK (Santa Cruz). Rabbit anti-p130Cas was obtained from A. Bouton at the University of Virginia Medical School, and a monoclonal paxillin antibody was obtained from C. Turner at SUNY Upstate Medical Center. The biotinylated polyclonal goat anti-mouse and biotinylated polyclonal rabbit anti-goat secondary antibodies were both purchased from DakoCytomation. Alexa Fluor 488 Phalloidin was purchased from Molecular Probes.

7.3 Recombinant protein production in *E. coli* (I-III) and in HEK 293T cells (III)

Expression and purification of GST-, His- and MBP-fusion proteins in *E. coli* and in HEK 293T cells were conducted according to the manufacturer's instructions (Amersham Biosciences, Qiagen and New England Biolabs, respectively). The purification of GST-SH2 fusion proteins (**I**) has been previously described (Mayer et al., 1991; Nollau and Mayer, 2001). After specific elution from their respective affinity beads, the proteins were concentrated. Then buffers were changed to desired ones by successive rounds of concentrating with Amicon Ultra-4 centrifugal filter columns (Millipore) or by dialyzing protein samples against phosphate buffered saline (PBS) in dialysis tubes (Spectrum Laboratories).

The tyrosine phosphorylation (**I**) of the PAK2 (aa 1-212)-His6 recombinant proteins was conducted in TKB1 bacterial cells, which harbor an activated EphB1 tyrosine kinase. The tyrosine phosphorylation was performed according to the manufacturer's instructions (Stratagene).

The protein concentration was assayed using the Lowry method (DC Protein Assay, BioRad), and the integrity of the purified proteins was assessed by separating samples on SDS-PAGE gels and staining with Coomassie blue.

7.4 Mammalian cell culture and transfections (I,III)

Human embryonic kidney fibroblast cells (HEK 293T) were purchased from ATCC and human osteosarcoma U2OS cells were obtained from Pekka Lappalainen, (University of Helsinki, Finland). Human epithelial carcinoma cells (A431) and human hepatoma cell line HepG2 expressing PDGFRβ were obtained from A. Kazlauskas (Schepens Eye Research Institute, Boston, USA). Mouse fibroblast NIH-3T3 cells (**I**) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-Alanyl-L-glutamine (Biochrom) and 1% penicillin-streptomycin (Sigma-Aldrich). NIH-3T3 cells stably expressing v-Abl, v-Src, v-Fps, CrkI or CrkII, and mouse fibroblast cells deficient in Src, Yes and Fyn (SYF cells) (**I**) were cultured in DMEM supplemented with 10% FCS. The MR20 human myeloma cell line (obtained from B. Lin and K.C.

Anderson, Dana-Farber Cancer Institute, Boston, USA) was maintained in RPMI supplemented with 10% fetal bovine serum (FBS).

Transient transfections of 293T cells were performed using the TurboFect reagent (Fermentas) or by Ca-phosphate precipitation methods (Manninen et al., 1998) for the production of recombinant proteins in the overlay assays. U2OS cells were transfected with the TransIT-LT1 transfection reagent (Mirus) according to the manufacturer's instructions. After the transfections, the cells were incubated for 20-48 h depending on the cell type and the transfected construct.

7.5   Immunoprecipitation (I,III)

Cells were washed once with PBS and lysed in the *in vitro* kinase assay (IVKA) lysis buffer (**III**) (50 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA and 1.5 mM $MgCl_2$) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and a Complete protease inhibitor cocktail (Roche). Cell samples for immunoprecipitations (**I**) were lysed in kinase lysis buffer (KLB) (150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 5 mM ethylene diamine tetraacetic acid (EDTA), 1 mM PMSF, 1% Triton X-100, 10% glycerol, 0.1% sodium pyrophosphate, 10 mM β-glycerophosphate, 10 mM NaF, 5 µg/ml of Aprotinin (purchased from Sigma) and 50 µM pervanadate). Lysates were precipitated with anti-Flag or anti-c-Myc antibodies (**III**) by using protein A-sepharose CL-4B beads (GE Healthcare) or with protein G-conjugated agarose beads, respectively (**I**).

7.6   Western blotting (I,III)

For Western blotting, immunocomplexes and total protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes for subsequent immunoblotting with specific antibodies. Biotinylated secondary antibodies and streptavidin-horseradish peroxidase were prior to enhanced chemiluminescence (ECL).

7.7   Luciferase reporter assay (III)

Cells were grown in a 24-well format and they were lysed in Luciferase Cell Culture Lysis Reagent (Promega) two days post-transfection. Lysates were cleared by centrifugation (5 min at 13,000 x g), and portions of the lysates were used for beta-galactosidase assays and for luciferase assays using the Luciferase Assay Reagent (Promega). Luciferase measurements were corrected based on the transfection efficiency. Data significance was determined using a Student's t-test.

7.8   SH2 domain studies (I)

7.8.1   SH2 Rosette assay

For the SH2 Rosette assay (Figure 11), A431 cells, wild-type or transformed (stably expressing viral oncogene products (v-Abl, v-Src and v-Fps) or SH3/SH2 adaptors (CrklI and CrklII)) NIH-3T3 cells were homogenized in KLB buffer. HepG2 cells were treated with or without pervanadate (50 mM for 30 min) prior to homogenization in KLB buffer. Insoluble material was removed by centrifugation. The protein concentration was determined using the Bradford assay (Bio-Rad).

Multiple whole-cell lysate samples were spotted on a nitrocellulose filter (>500ng sample / spot) placed in a well of a 96-well chamber plate. SH2 binding and sample washing were performed in the chamber plate, and the binding was detected by chemiluminescence and quantified by densitometry. Quantified values for each probe were compared to the positive control (lysate from 3T3 cells expressing v-Abl).

The results from the SH2 Rosette assay were validated using an anti-actin antibody to evaluate interspot variation (accuracy of spotting), an anti-phospho-tyrosine antibody to investigate the tyrosine phosphorylation status and avidin to check the biotinylation of the protein samples.
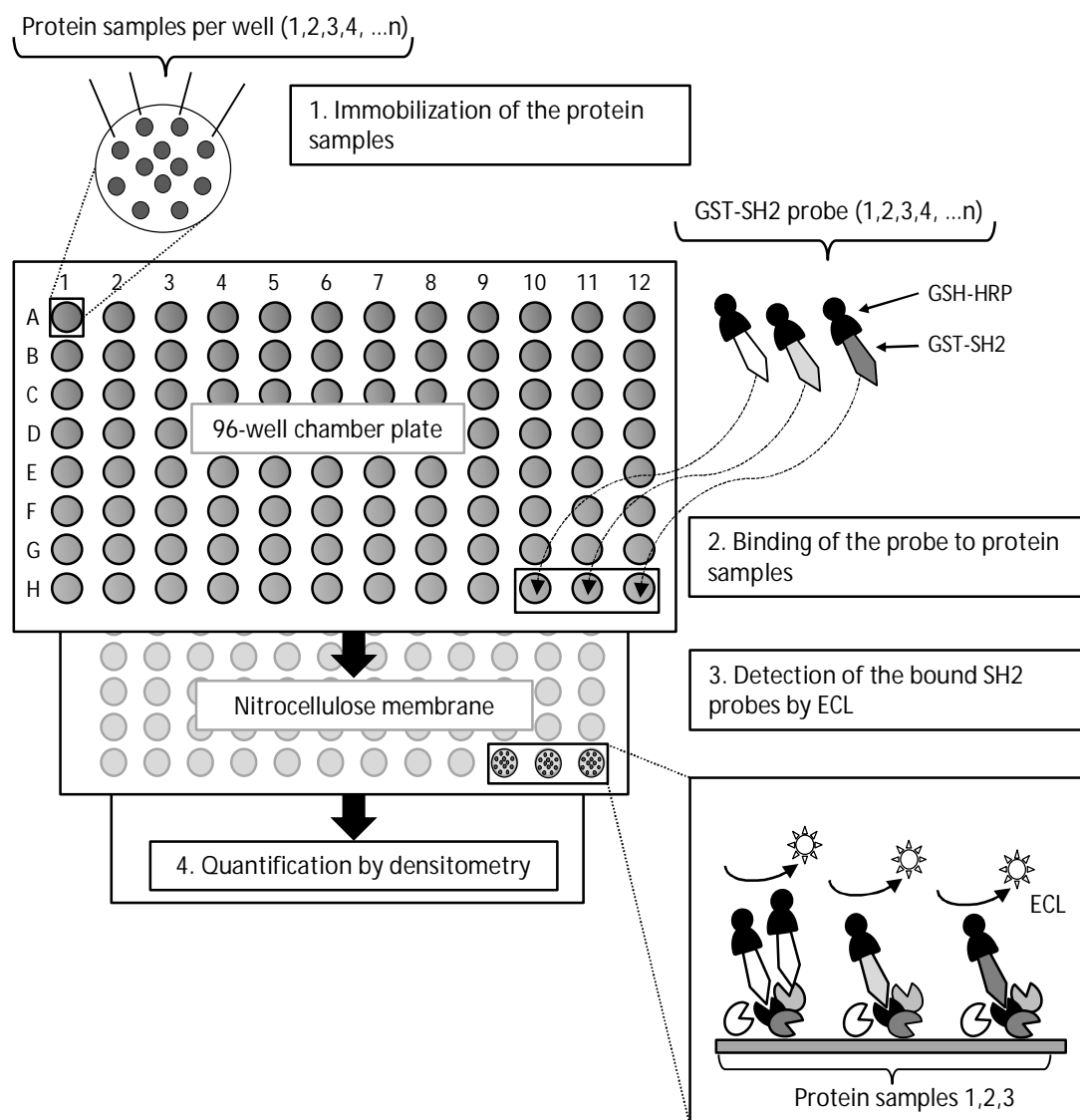
Figure 11. The principle of Rosette assay. Protein samples (up to 12 in this example) are immobilized onto the nitrocellulose membrane in placed in a well of a 96-well chamber plate. Duplicate sample arrays are spotted in multiple wells, and each GST-SH2 probe coupled to glutathione-horseradish peroxidase (GSH-HRP) is allowed to bind to a set of protein spots in separate sample wells. Binding of SH2 probes to protein samples is detected by chemiluminescence and quantified by densitometry. Modified from the original communication **I**.

7.8.2   1D Far-western blotting

This method was used to confirm and expand the Rosette assay results. Unphosphorylated and tyrosine-phosphorylated purified recombinant proteins, in the presence or absence of pervanadate-treated wild-type and transformed NIH-3T3 cells as well as adherent and suspended c-Src reconstituted SYF cell lysates, were separated via SDS-PAGE gels, transferred onto nitrocellulose membranes and then probed with biotinylated SH2-domain samples.

7.9   SH3 domain studies (II)

7.9.1   Production of the phage library

The multivalent phage library containing human SH3 domains was constructed using the pG8H6 phage vector. The phagemids were electroporated into *E. coli* TG1 cells. The individual colonies were pooled and grown overnight at 30°C in 2x YT media containing 100 µg/ml ampicillin and 2% (w/v) glucose (2x YT-AG). Cultures were diluted 1:10 in 2x YT-AG and infected with M13KO7 helper phage ($5 \cdot 10^8$ pfu/ml, Amersham Biosciences) at 37°C. After 2 h, cells were pelleted, and the media was replaced with 2x YT media containing 100 µg/ml ampicillin and 50 µg/ml kanamycin (2x YT-AK). The double-resistant cultures were grown overnight at 37°C, and the supernatant containing the infectious recombinant phages was collected and passed through a 0.45 µm filter. Phage titers were normalized to 1×10^{11} infectious units per milliliter and stored in aliquots at 4°C.

7.9.2   Phage selection

Six-well plates were coated with 10-20 µg/ml GST- PAK2 (aa 1-212), GST-Nef, GST-ADAM15 or GST in sodium carbonate (50 mM pH 9.6) at 4°C overnight. The wells were blocked with 5% milk in PBS containing 0.05% Tween 20, and the wells were washed briefly with PBS before the addition of recombinant phages (1×10^{11} pfu per well). The plates were then incubated for 2 h at room temperature (RT). After incubation with the phages, the wells were washed three times (5 min each)

with PBS containing 0.05% Tween 20 and two times with PBS. After washing, TG1 cells, which had been cultured in 2x YT until they reached log phase, were added directly to the washed wells. After incubation for 2 h at 37°C, 1% of the sample was removed and plated on ampicillin plates to determine the infectious titer of the selected phages. These plates also served as indicators for enrichment of specific clones when the plates were compared to plates infected with phages from GST-coated wells processed in parallel. The remaining infected bacteria were supplemented with 100 µg/ml ampicillin and 2% glucose and subjected to a second infection with M13KO7 helper phages ($5 \cdot 10^8$ pfu/ml) for 2 h at 37°C. After this second infection, cells were pelleted and resuspended in equal volumes of 2x YT-AK. After an overnight incubation, the amplified recombinant phage supernatants were collected as described above and used for a subsequent round of selection. After two rounds of selection, 12 colonies were picked to prepare phagemid DNA, and the SH3 inserts were sequenced on an ABIPrism 310 sequencer (Applied Biosystems). An overview of the phage display screen for assaying human SH3 proteome for binding to target proteins of interest is shown in Figure 12.

7.9.3 Recombinant protein binding assay for SH3 domains

Purified recombinant His-tagged or MBP-fusion proteins (**II**) were used to coat MaxiSorp F8 strips (Nunc), and the strips were blocked with 1.5% BSA in TBS at room temperature. The wells were then washed three times with washing buffer (WB). GST-fused SH3 domains were conjugated to horseradish peroxidase following a 1 h incubation on ice with glutathione-horseradish peroxidase (Sigma-Aldrich). After incubation, the SH3 domains were serially diluted in TBS containing 1.5% BSA and 2 mM dithiothreitol and then added to wells. After washing three times with WB, 50 µl of the substrate reagent (2,2'-azino-bis (3-ethylbenzthiazoline)-6-sulphonic acid; (Sigma)) was added to each well. Enzymatic reactions were stopped after 10 min with 50 µl of 1% SDS, and the optical density was measured at 405 nm.

When the interactions were strong ($K_D < 0.5$ µM), the data points from serial SH3 dilutions were plotted on Scatchard plots, and the apparent K_D values were derived from the slopes. To model the weaker interactions, data was assigned to two

broad categories based on estimated affinities in the range of 0.5-3 or 3-20 µM. Interactions that were weaker than the Nef/Fyn-SH3 interaction (K_D 15.8 µM; (Arold et al., 1998) or those that were too weak to be detected at a concentration of 38 µM were assigned to a final category (defined by a K_D value >20 µM).
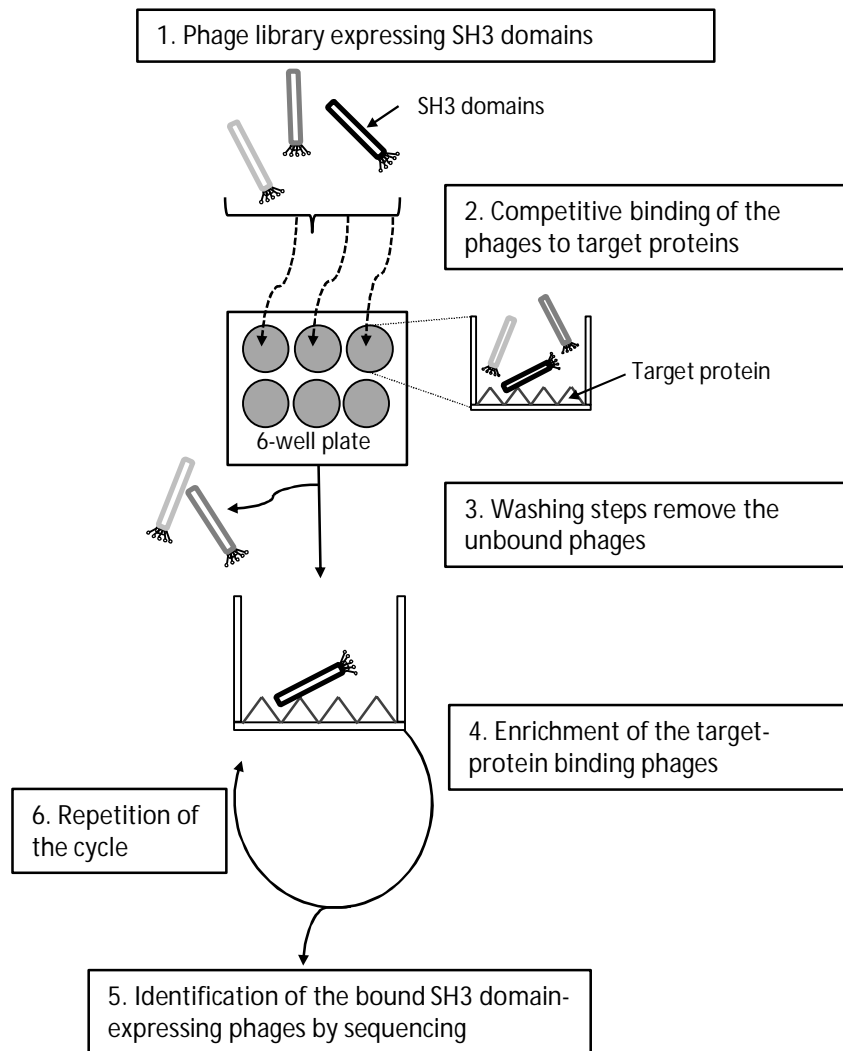
Figure 12. Overview of the SH3 domain screen to identify target proteins by phage display. Protein samples are immobilized on six-well plates such that each target protein is bound to one well. A bacteriophage library expressing SH3 domains is allowed to compete for binding to each target protein. Washing steps remove the non-specifically bound phages. The target-protein-bound phages are amplified by infection and multiplication in *E. coli* to generate enriched phages. This cycle is repeated twice. After each round of panning, the target protein-binding phages are identified by sequencing.

7.10 Microscopy / Immunocytochemistry (III)

To detect immunofluorescence, U2OS cells were grown on coverslips in 6-well plates. The cells were fixed 18-24 h post-transfection with 3.7% formaldehyde in DMEM supplemented with 10% FCS for 20 min at 37 °C. Cells were then washed three times with PBS and permeabilized with PBS containing 0.5% Triton X-100 and 10% FCS for 15 min at RT. After blocking with PBS containing 3% BSA and 0.05% Tween 20 for 1 h at RT, the cells were incubated with primary antibodies or Alexa Fluor 488 Phalloidin (for F-actin staining) at the recommended concentrations. Primary antibodies were incubated for 1 h at RT in PBS containing 10% FCS, and Alexa Fluor 488 Phalloidin was incubated for 20 min at RT in PBS. Cells were washed three times with PBS and mounted on slides using the ProLong Gold Antifade reagent with DAPI (4,6-diamino-2-phenylindole; Invitrogen). Images were acquired using confocal microscopy on an inverted Olympus IX70 microscope coupled to Andor iQ software (Andor Technology).

8. RESULTS

8.1 High-throughput phosphotyrosine characterization using SH2 domains (I)

8.1.1 Characterization of SH2 domains

To generate a comprehensive collection of SH2 domains, cDNAs encoding known SH2 domains were cloned into bacterial vectors and fused to GST. A collection of 110 SH2 domain constructs represented 95% (114/120) of all human SH2 domains (and 104 out of the 110 SH2 domain-containing proteins) (Liu et al., 2006). The solubility of GST-SH2 domains was investigated in small-scale experiments, and the SH2 domains were categorized into three groups based on solubility (good, moderate and poor) (**I**, Figure 1A). Approximately half of the GST-SH2 domains were highly soluble. Poorly soluble SH2 domains were associated with certain SH2 domain families (**I**, Figure 1A), including JAK, STAT and suppressor of cytokine signaling (SOCS) protein families, and have been described by others (Babon et al., 2006; Jones et al., 2006).

The phosphotyrosine-dependency of SH2 domain binding was determined by GST-SH2 pull-down assays followed by immunodetection and by far-Western blotting of cell lysates (**I**, Figures 1A and 1B). All evaluable GST-SH2 domains exhibited clear pTyr-dependent binding (**I**, Figure 1A).

The protein binding specificity of 74 human SH2 probes with adequate solubility and low non-specific binding was determined by far-Western blotting experiments. To assess similarities in the binding patterns of various SH2 probes, the signals on the blots were quantified, and the data were used in clustering analyses to quantitatively measure overall binding similarities (**I**, Figure 2A). SH2 domains from closely related families, such as Nck (Nck1 and Nck2) and Grb (Grb2, Grab and Grab2) (**I**, Figure 2A, clusters 1 and 2) demonstrated similar yet unique binding patterns. About one third of the SH2 domains (including almost all nonreceptor

tyrosine kinases) clustered in a group of broad binding specificity (**I**, Figure 2A, cluster 3). SH2 domain relatedness based on primary sequence and binding patterns was also investigated (**I**, Figure 2B). This generally resulted in rather poor correlation. In some cases, there were apparent contradictions between binding patterns and sequence similarities. For example, the four closely related tensin family members (Tensin/Tns1, Tenc1, Cten/Tns4 and Tem6/Tns3/Tens1) each exhibited distinct binding patterns. In contrast, we found that the nucleosome assembly protein 1 like 4 (Nap4, also called Socs7) SH2 domain showed very similar binding pattern in comparison to Src family SH2 domains, although these SH2 domains are only distantly related (**I**, Figure 2B).

8.1.2   SH2 profiling for phosphoproteins

A reverse-phase assay, dubbed the Rosette assay, was developed to study the binding of SH2 domains to protein samples (**I**, Figure 3 and Figure 11). Here, multiple protein samples were spotted onto nitrocellulose filters in a 96-well chamber plate, and these sample arrays were allowed to interact with each SH2 probe; and the binding of SH2 probes was detected and quantified.

The SH2 Rosette assay was utilized to screen PECAM-1 peptides. Peptides containing either of the two ITIM motifs of PECAM-1 were synthesized in the phosphorylated (pTyr663 and pTyr686) and unmodified (Tyr663 and Tyr686) forms. A panel of SH2 domain probes demonstrated different binding preferences, but they all showed no binding to the unphosphorylated peptides (**I**, Figure 4). The SH2 Rosette assay detected almost all SH2 domains (SHP1, SHP2, Src, Fyn, PLCγ1, SHIP, PI3K (P85α)) that have been reported to bind phosphorylated PECAM-1. The N-terminal SH2 domain of SHP2 (also called PTP1D or PTPN6) preferentially bound pTyr663, whereas the tandem SHP2 SH2 probe (Shp2NC) bound to both pTyr663 and pTyr686. Moreover, previously unappreciated interactions were also detected. The C-terminal Src kinase (Csk) and Sap SH2 domains accounted for the strongest binding to pTyr686.

A cocktail of SH2 domains were used in the Rosette assay format to profile the binding preference of the N-terminal PAK2 regulatory domain (PAK2N), which includes three tyrosine phosphorylation sites (Y130, Y139 and Y194) (**I**, Figure

5A). Most of the SH2 probes exhibited weak binding to PAK2N; however, the Sap SH2 domain showed strong PAK2N binding (**I**, Figure 5B). The Sap SH2 domain and its close relative, Eat2, were used in a far-Western approach to further identify the preferred binding sites in PAK2N by mutating one or all three tyrosines in PAK2N to phenylalanine. The PAK2N Y194F mutation abolished the binding of Sap SH2 (**I**, Figure 5C). Conversely, Eat2 showed similar binding to all three PAK2N proteins containing two phosphorylated tyrosines.

8.1.3 Profiling of entire proteomes and adhesion-dependent cellular responses by SH2 domains

Following the Rosette assay, signals were quantified to profile the SH2 binding site level similarities in v-Abl, v-Src and v-Fps oncogene-transformed cell lines *in vivo*. v-Abl, v-Src and v-Fps-transformed NIH 3T3 cells (**I**, Figure 6A) could be distinguished by their binding preferences for single SH2 domains. Weak binding was observed, for example, for the Fer SH2 binding to v-Abl lysates (compared to other lysates). Weak interactions were also observed for Grb2 and Grab2 SH2 domains binding to v-Src lysates and for Emt and Nck SH2 domains binding to v-Fps lysates (**I**, Figure 6A). To confirm and extend the Rosette assay results an analytical far-Western assay was used. Non-receptor tyrosine kinases bound to a variety of phosphorylated proteins in cell lysates, whereas the Grb2 adaptor protein family showed limited binding to phosphoproteins (**I**, Figure 6B). The binding preferences of the SH2 domains used for the far-Western approach correlated well with the results of the Rosette assay (**I**, Figure 6B).

To test the approach of profiling cells under different physiological conditions, the SH2 domain binding profiles of adherent cells were compared to the profiles of suspended cells using Rosette assay (**I**, Figure 7A). Src family kinases are activated upon cell adhesion and mediate cell survival and cell proliferation (Giancotti and Tarone, 2003). SYF cells (mouse embryonic fibroblasts), which lack the Src, Yes and Fyn family kinases, and SYF cells re-expressing c-Src (SYF-Src) were used to observe Src family kinase-dependent phosphorylation events stimulated by cell adhesion. In this approach, SYF cells exhibited lower global tyrosine phosphorylation levels compared to SYF-Src cells under all conditions. Moreover, adherent SYF-Src cells had higher global tyrosine phosphorylation levels when

compared to suspension cells. Overall, nearly all SH2 domains exhibited increased binding in adherent cells, and approximately one fourth (11/45) of the analyzed SH2 domains showed a two-fold increase in binding following cell adhesion (**I**, Figure 7A, asterisks).

A far-Western approach was utilized to study the SH2 binding sites, which were highly adhesion-dependent (**I**, Figure 7B). The SH2 domains showed distinct binding patterns to cellular proteins. For example, the Src SH2 domain exhibited binding to various phosphorylated proteins, which was similar to an overall anti-pTyr binding profile. However, the Csk and Cten SH2 domains bound each to single bands of approximately 70-80 kDa and 130 kDa, respectively, in an adhesion-dependent manner.

To identify adhesion-dependent SH2 binding proteins, antibodies against proteins previously described in cell adhesion were used. FAK, p130Cas and paxillin were immunoprecipitated, and their tyrosine phosphorylation levels in adherent and suspended cells were determined (**I**, Figure 7C). In every case, phosphorylation was enhanced under adhesion conditions. Moreover, individual SH2 domains were used to investigate the binding specificity of FAK, p130Cas and paxillin. The SH2 binding profiles could be categorized into five groups (**I**, Figure 7E) based on their binding preference for these three proteins. SH2 domains, such as Src, Fyn and Arg exhibited similar binding pattern for all three of these proteins upon adhesion, while some SH2 domains exhibited more specific binding pattern. For example, the Nck and Cten SH2 domains only bound p130Cas, and the Csk SH2 domain only bound to paxillin.

8.2   Identification of preferred SH3-mediated interactions by human proteins (II)

8.2.1   Human SH3 proteome phage library

To identify the complete and non-redundant collection of human SH3 domains, BLAST searches against sequence databases using representative SH3 domains were performed. Human SH3 domain sequences found in the SMART and PFAM databases were also analyzed. The SMART and PFAM algorithms assigned the

amino-terminal boundaries similarly, whereas carboxy-terminal assignment by SMART and PFAM was modified for a few atypical SH3 domains to preserve continuity of the sequence across the last two β-strands. Structural studies of post-synaptic density protein 95 (PSD-95) indicated that the βF strand of the PSD-95 SH3 domain separated from the rest of this SH3 domain by a guanylate kinase-like domain (McGee et al., 2001). Therefore, due to this unique split domain organization, it seems that some SH3 sequences in the MAGUK family in this collection are lacking the last β-strand of the proper SH3 fold.

In cases where only a single amino acid varied in the same SH3 domain, only the most prevalent sequence was added to our collection. Conversely, splice variants, which differed in the lengths of the loop regions between SH3 β-strands, were considered as independent SH3 domains. Altogether, our collection was completed with such splice variants and contained 296 different SH3 domains. Some proteins contain up to six SH3 domains; thus the total number of human genes encoding these SH3 domains was 217.

To study the properties of individual human SH3 domains, codon-optimized genes encoding the different SH3 domains were cloned into a phagemid vector and fused to a cDNA encoding the pVIII M13 coat protein. These phagemids were transformed into *E. coli*, and supernatants with recombinant phages were subsequently mixed in equal ratios to produce an infectious library containing all SH3-displaying phages at the same titer.

8.2.2   SH3 domains selected by Nef

Nef was used to select our multivalent SH3 library, and increased binding of phages to Nef was observed compared to the plain GST as a control (**II**, Figure 1). The identity of the Nef-selected phagemids was determined, and all of the enriched clones (more than 30) contained the Hck SH3 domain.

To determine whether Nef could select other SH3 domains if phages containing the Hck-SH3 domain were excluded from the library, we prepared a similar SH3 phage library that expressed all SH3 domains except Hck and related Lyn SH3 domains. Use of this library resulted in the selection of heterogeneous populations of SH3 domains, of which certain SH3 domains, including the third SH3 domain of

Tuba, the second SH3 domain of the adapter protein Crk and the SH3 domain of a predicted protein dubbed 7A5 (also called metastasis-associated in colon cancer protein 1, MACC, NP_877439), were found (see Table 2). The outcome of the experiment was variable, indicating that the SH3 domains in the library lacking Hck and Lyn did not bind Nef with sufficient affinity.

8.2.3 SH3 domains selected by PAK2

The identity of SH3 phages that preferentially bound PAK2 was determined after two rounds of affinity selection. 17 clones from two independent experiments using the complete library revealed that most (13/17) of these SH3 domains belonged to β-PIX. The remaining four clones were identified as α-PIX, vinexin-III/III (III/III denotes the third of the three SH3 domains in this protein) and two copies of an SH3-containing predicted protein dubbed FLJ00204 (see Table 2). FLJ00204 was found to be part of a larger gene product that could be assembled *in silico*. We renamed this novel protein POSH2 because of its close similarity to a previously characterized protein called POSH (Tapon et al., 1998). Like POSH, POSH2 contains four SH3 domains, and the third SH3 domain (III/IV) was selected by PAK2 in the phage display screen.

Because the PIX-family SH3 domains were predominantly found in the PAK2-selected phages, we generated a phage library that did not include the α- and β-PIX. Half of the 29 clones from two independent experiments with this PIX-negative library contained Nck1-II/III. The second most prevalent clone was ponsin-III/III, which was found five times. ArgBP2-III/III was found twice and vinexin-III/III three times. Notably, ponsin, vinexin and ArgBP2 are structurally and functionally related to each other (Kioka et al., 2002), and thus, altogether the third SH3 domain of these protein family members accounted for one third (10/29) of the clones. The remaining SH3 domains (one of each) were identified as Src, Hck, Grb-I/II and POSH2-III/IV. In summary, PAK2 selected both novel (such as the POSH2 protein and ponsin-vinexin-ArgBP2 protein family) and also well-established PAK-interacting proteins (such as PIXs, Grb2 and Nck1).

8.2.4   SH3 domains selected by ADAM15

The intracellular region of ADAM15 was fused to GST and used as a target protein. The identity of 18 clones (after one round of selection) and another 16 clones (after two rounds of selection) was determined (see Table 2). In the first round selection, SH3 domains from nephrocystin (5/18) and from a predicted protein dubbed MGC32065 (11/18) predominated. Both of these are novel ADAM15-ligands. Two other clones were identified as the N-Src and Fish-I/V SH3 domains. Nephrocystin and MGC32065 were almost exclusively found after the second round of selection (6/16 and 9/16, respectively). Moreover, MGC32065 contains both PX and SH3 domains, and it is significantly homologous to the sortin nexin family. SNX9/SH3PX1 also belongs to this family, and has been shown to bind ADAM15 (Howard et al., 1999). Human sortin nexins named SNX1-27 could be found in the databases, but MGC32065 was renamed SNX30 because mouse genes dubbed up to SNX28 and SNX29. Subsequently, the same protein was discussed in a study by Seet and Hong and they re-named it SNX33 (Seet and Hong, 2006).

8.2.5   Biochemistry of the interactions

To confirm that the selected SH3 domains bound to their target proteins with high affinity and specificity, a semi-quantitative protein interaction assay was used (**II**, Figure 2). For this assay, 22 SH3 domains were chosen and the binding of these SH3 domains to the target proteins was investigated (**II**, Figure 2). These SH3 domains included the domains identified in the screen and their homologues in addition to the SH3 domains that are known to interact with Nef, PAK2 and ADAM15. This created a panel of 66 measured interactions (**II**, Figure 3).

For the stronger interactions (K_D < 0.5 µM), the K_D values derived from the Scatchard plot slopes correlated well with published K_D values for the PAK/β-PIX (24 nM) (Manser et al., 1998) and for the Nef/Hck (250 nM) complexes (Lee et al., 1995) (**II**, Figure 2). The Scatchard plot analysis of weaker interactions was problematic; thus, these interactions were categorized into two groups based on their estimated binding affinities in the range of 0.5-3 and 3-20 µM. Interactions that were too weak to be detected when using 38 µM of the GST-SH3 domain or

interactions that were weaker than the Nef/Fyn-SH3 binding (K_D 15.8 µM) (Arold et al., 1998) were placed in the remaining category (K_D value > 20 µM).

SH3 domains that were included in the analysis based on their selection from the screen, bound to their ligands with high affinity. Moreover, the SH3 domains that bound to Nef, PAK2 and ADAM15 with the strongest affinities were also identified in the phage display screen as the preferred SH3 partners for these ligands. Strong interactions appear to be highly specific. Only modest or no binding was observed for example for the interaction between the vinexin family and POSH family SH3 domains to ADAM15 or Nef, or for the interaction between SNX30 or nephrocystin and Nef or PAK2, despite the presence of ideal SH3 binding sites in these target proteins.

Table 2. Summary of the SH3 domains selected by Nef, PAK2 and ADAM15 in the phage display screen. Roman numbers indicate which SH3 domain in the particular protein bound to the target protein. Moreover, the percentages of the selected SH3 domains are shown (for details see text). Phage libraries expressing different sets of SH3 domains are also indicated.

		Target protein		
		Nef	PAK2	ADAM15
SH3 domains interacting with target protein	library expressing all SH3 domains	Hck 100%	β-PIX ⎤ 76,5% α-PIX ⎦ vinexin-III/III ⎤ 23,5% POSH2-III/IV ⎦	nephrocystin 32,6% SNX30 58,4% N-Src ⎤ Fish-I/V ⎦ 8,7% (1st round of selection)
	library without Hck and Lyn SH3 domains / library without β-PIX and α-PIX SH3 domains / library expressing all SH3 domains	Tuba-III/IV Crk-II/II MACC	Nck1-II/III 51,7% ponsin-III/III ⎤ ArgBP2-III/III ⎥ 34,5% vinexin-III/III ⎦ Src Hck ⎤ Grb2-I/II ⎥ 13,8% POSH2-III/IV ⎦	nephrocystin ⎤ 93,4% SNX30 ⎦ (2nd round of selection)

8.3   Characterization of the novel POSH2 protein (III)

### 8.3.1   Cloning and identifying a new POSH family member

In our screen using all human SH3 domains to identify binding partners of PAK2 (**II**), we found an SH3 domain of a novel protein and further characterized this protein. We re-named the novel protein POSH2 because of its close similarity to POSH1 scaffold protein (Tapon et al., 1998). Subsequently, Wilhelm and colleagues identified a similar protein in rats via BLAST homology searches using POSH1 sequence and they named this protein POSH2 (Wilhelm et al., 2007a). The identified protein was not an ortholog of human POSH2, and due to this disparity, we refer to the Wilhelm protein as POSH3.

The POSH2 protein sequence (Appendix 2) was first constructed *in silico*, and the corresponding cDNA was subsequently cloned), resulting in a full-length coding sequence of 882 amino acids and a predicted molecular weight of 93 kDa. Analysis of the POSH2 protein sequence revealed a potential zinc RING finger structure (aa 57-97), four SH3 domains (aa 197-252, aa 259-317, aa 467-524 and aa 826-882) and a minimal Rac-binding site (aa 369-439) (Figure 13 and Appendix 2), which was previously described in POSH1 (Tapon et al., 1998). POSH2 is highly conserved in mammals. Based on the microarray expression data of human POSH2 (available from Weizmann Institute of Science GeneNote service), POSH2 is expressed ubiquitously in different tissues (thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, and prostate).

Further sequence comparison of POSH family members revealed that *POSH2* did not have an in frame stop codon preceding the conserved methionine start codon. Conversely, POSH2 had another conserved in frame upstream translation initiation start site (ATG), and our BLAST searches revealed that the translated amino acid sequence was present in POSH2 sequences in different species (**III**, Figure 1). Because neither of the sequences surrounding either ATG start codons were perfect Kozak consensus sequences for translation initiation, we expressed GFP-tagged N-terminal POSH2 fragments to determine which ATG was preferred as the translation start site. Western blot and quantification analysis indicated that the first upstream translation initiation site was preferred. Additionally, the role of the POSH2 N-terminal extension region in cellular localization was studied, but differences in

72

cellular distribution between the shorter and the longer translated POSH2 fragments were not observed, suggesting that the N-terminal extension does not act as a targeting sequence (Appendix 1).
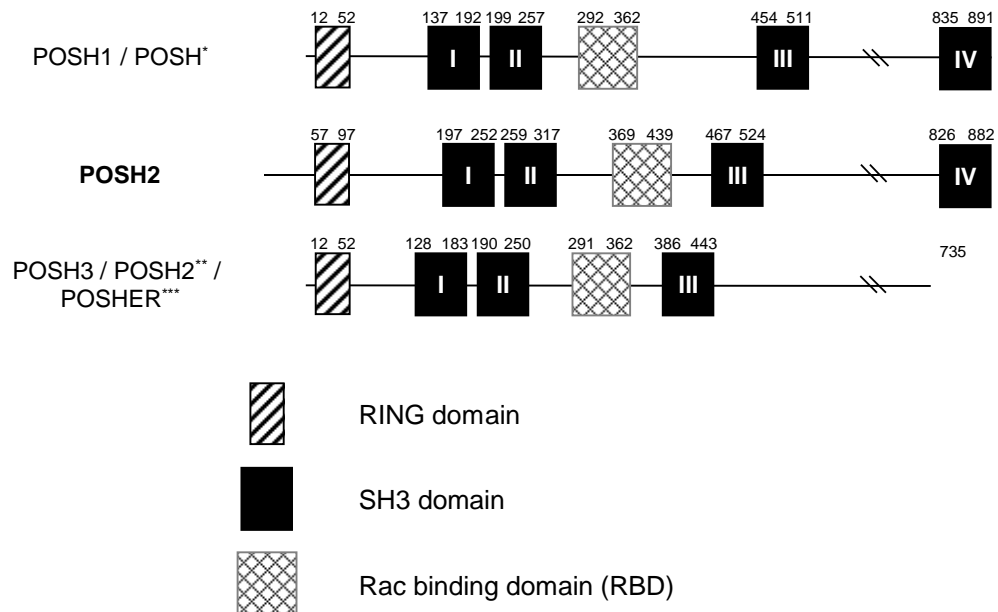
Figure 13. Domain structures of the POSH protein family. Representative structures of vertebrate POSH proteins are shown. The POSH2 protein described in our study is shown in bold. The various nomenclature of POSH proteins given by other authors is indicated by asterisks (*(Tapon et al., 1998), **(Wilhelm et al., 2007a), ***(Wilhelm et al., 2012)). The region C-terminal to the third SH3 domain is not drawn to scale and is thus marked with two crossing lines.

8.3.2 POSH2 has a functional RING domain

Many of the RING domain-containing proteins can act as E3 ligases and to mediate ubiquitin transfer (Deshaies and Joazeiro, 2009). POSH2 contains a characteristic RING finger domain ($Cys_3HisCys_4$), and a mutational approach was used to investigate whether the RING finger domain of POSH2 possessed E3 ligase activity. The RING finger domain has eight metal-binding residues that coordinate to bind two zinc atoms (Borden and Freemont, 1996). Two of the metal-binding amino acids in the RING domain were mutated (C28A and H30A for POSH1, described in (Kim et al., 2005) and C73A and H75A for POSH2 (see Appendix 2)). Transfection

studies revealed the transfection efficiency, which measures both the survival of the transfected cells as well as protein expression. In these studies, we found a significant reduction in the transfection efficiency of POSH1 and POSH2 when ubiquitin was over-expressed in these cells. This suggested that a significant number of the transfected cells had undergone apoptosis (**III**, Figure 2). Despite the low expression of wild-type POSH proteins compared to their RING domain-mutated counterparts, wild-type POSH1 and POSH2 were highly ubiquitinated, as evidenced by a smear of signal at and above the site of POSH proteins mobility. This confirms the hypothesis that these amino acids (C28A and H30A for POSH1 and C73A and H75A for POSH2) are crucial for POSH protein self-ubiquitination. When the RING domains of POSH proteins were mutated, the POSH protein expression was increased in the presence of ubiquitin. However, we did not observe a consistent difference between the wild-type and RING domain-mutated proteins in the absence of overexpressed ubiquitin, which is inconsistent with data presented for POSH1 and POSH3 (Wilhelm et al., 2007a; Xu et al., 2003).

8.3.3 POSH2 partial CRIB domain affects JNK activation

POSH1 is a Rac1-binding protein and contains a minimal Rac-binding region that encompasses 70 amino acids (aa 292-362) (Tapon et al., 1998). Sequence alignment of POSH proteins (Appendix 2) indicated that the Rac-binding site in POSH1 was highly homologous to the site in POSH2; thus, we investigated whether POSH2 possessed GTPase-binding activity in this region (aa 369-439). In the GTPase overlay assay, POSH2 was observed to interact only with the GTP-bound form of Rac1, and no interaction between POSH fragments and Cdc42 was detected (**III**, Figure 3A). These results are consistent with the results obtained using the POSH1 Rac-binding domain (Wennerberg et al., 2002).

POSH1 contains a unique Rac-binding domain, and the domain sequence had no similarities compared to sequences of other GTPase-binding proteins (Tapon et al., 1998). However, in our alignment of POSH1-3 sequences, we observed that the Rac-binding region is conserved among these proteins (**III**, Figure 3B) and found that a portion of the Rac-binding region (I-S-X-P) has similarities to the CRIB domain consensus sequence I-S-X-P-$(X)_{2-4}$-F-X-H-X-X-H-V-G (Burbelo et al.,

1995). The CRIB domain of PAK kinase is important for binding to p21 GTPases (Zhao et al., 1998). In this study, the ISP amino acids within the PAK1 CRIB motif (I75N, S76P and P78A) were mutated and these mutants were deficient in binding to Cdc42 (Zhao et al., 1998). As a result of these observations, we produced POSH2 fragments that were mutated within the putative CRIB domains (I403N, S404P and ISP [I403N, S404P and P406A]) and compared these mutants to POSH2 control mutants (L385A and P414A). The control mutants contained amino acid changes located on either side of the I-S-X-P sequence. Our ability to detect Rac1 binding was close to the limit of the detection for the GTPase overlay assay, thus, we used a more sensitive pull-down assay to study the interaction of our mutants with the GTPase. In this pull-down assay, POSH2 fragments and dominantly GTP-loaded Rac were co-transfected into cells, and the binding of the POSH2 to Rac1 was detected. Mutations (I403N, S404P and ISP) in the I-S-X-P region decreased the binding of POSH2 to Rac1 when compared to Rac1 binding by the wild type POSH2 fragment and the POSH2 control mutants (**III**, Figure 3C). These results implied that the POSH2 I-S-X-P sequence functions as a partial CRIB domain and is crucial for binding Rac1.

The POSH1 and POSH3 scaffold proteins have been described to enhance JNK activation (Tapon et al., 1998; Wilhelm et al., 2007a). In addition, POSH1 has been shown to induce c-Jun phosphorylation and to increase AP-1 reporter activity (Wang et al., 2007). To test our hypothesis that the highly homologous POSH2 protein could induce JNK activation, we co-transfected wild-type POSH2 and an AP-1 luciferase reporter construct into 293T cells to measure JNK activation. We demonstrated that wild-type POSH2 increased AP-1 activity in the reporter assay, supporting the model that POSH2 promotes JNK activation (**III**, Figure 3D). To further test the role of the POSH2 partial CRIB-like motif in JNK activation, the S404P CRIB-mutant POSH2 was used. This mutant induced weaker AP-1 activation when compared to wild-type POSH2 (**III**, Figure 3D).

9. DISCUSSION

9.1 Proteome-wide analysis of SH2 domains

Tyrosine phosphorylation controls various cellular responses, such as cell survival and differentiation (Hunter, 2000; Hunter, 2009). Many tyrosine phosphorylation sites exert their downstream signaling effects by creating binding sites for SH2 domains. Therefore, we can gain valuable information on the activation state of the cell by profiling the binding activity of SH2 domains. We generated a high-throughput, quantitative SH2 profiling approach (Rosette assay) and used this assay to decipher *in vivo* SH2 interactions and signaling pathway activity in the cell. This reverse-phase Rosette assay provided comprehensive and quantitative SH2 domain binding data by using small amounts of sample in a dot-blotting format. Additionally, this assay has a clear advantage with respect to sensitivity and applicability to many types of samples compared to prevailing methods used in characterizing the global tyrosine phosphorylation (Machida et al., 2003).

SH2 and PTB domains have been described as major binding modules that recognize tyrosine-phosphorylated proteins (Pawson and Nash, 2003; Schlessinger and Lemmon, 2003; Yaffe, 2002). Only about one fourth of the known PTB domains bind in a phosphorylation-dependent manner, and a majority of the PTB domains recognize non-phosphorylated ligands or phosphoinositidies (Schlessinger and Lemmon, 2003). Moreover, other protein interaction domains have been shown to interact in a pTyr-dependent manner, including C2 domain in PKCδ (Benes et al., 2005) and the recently discovered Hakai pTyr-binding domain (Mukherjee et al., 2012). However, these domains form much smaller or even single member proportions of the phosphotyrosine recognition domains. The SH2 domains used in our assays demonstrated increased binding following tyrosine phosphorylation, indicating the importance of the SH2 domain as a preferred pTyr-recognition module and as a primary element in tyrosine kinase signaling. These observations

also confirmed the validity of the assay, which was based on the use of SH2 domains to profile the pTyr status of the cell.

Previously described strategies exploiting random peptide libraries, peptide arrays or MS-based approaches identified preferred specific binding sites for SH2 domains (Blagoev et al., 2003; Hwang et al., 2002; Songyang et al., 1993). However, these experiments do not provide information on the abundance of the binding sites in the cell. Using a panel of SH2 domains in a far-Western approach, we found that SH2 domains of non-receptor tyrosine kinases bound to a large spectrum of sites in the cell, whereas other SH2 domains, such as those in the Grb2 adaptor protein family, recognize a smaller range of phosphoproteins. This is consistent with the general model, in which SH2 domains of non-receptor tyrosine kinases bind to a wide variety of phosphorylated ligands, thereby promoting processive phosphorylation of downstream effectors (Mayer et al., 1995). Conversely, SH2 domains of adaptor proteins are thought to exert different outputs by interacting with more specific combinations of binding partners (Pawson, 2004; Pawson, 2007).

Single SH2 domains have been generally thought to bind to a particular phosphorylation site regardless of their flexibility in binding specificity (Pawson et al., 2004). Although, SH2 binding preferences vary, some SH2 domains may bind to the same site, implicating that some SH2 domains share similar binding specificities. SH2 binding specificities were also assessed by comparing SH2 sequence alignment with their ligand-binding profiles. The correlation between binding patterns and evolutionary distance was poor. Similarly, Tinti and collaborators (Tinti et al., 2013) failed to identify a correlation between sequence homology over the entire domain and the recognition specificity of a peptide. These results are consistent with the SH2 domain structural studies, which have demonstrated that only a few residues in the SH2 domain determine the binding specificity and contact the ligand (Bradshaw and Waksman, 2002; Kuriyan and Cowburn, 1997). Therefore, only sequence differences in some regions of the domain have an effect on binding.

SH2 domains were also used to profile SH2 domain binding to the tyrosine phosphorylated PAK2. N-terminal tyrosine phosphorylation of PAK2 leads to superactivation of the kinase (Renkema et al., 2002). The authors showed that PAK2 tyrosine phosphorylation could be induced by Src kinases (such as Fyn); however,

the specific mechanism of superactivation is not yet known (Renkema et al., 2002). We found that the Sap SH2 domain probe bound strongly to PAK2N, and this binding event was dependent on the phosphorylation of PAK2 Y194, which is one of the three N-terminal tyrosines (Y130, Y139 and Y194). Sequences surrounding pY130 and pY139 do not match strictly the consensus; however, sequence near pY194 conforms reasonably well to the binding consensus of Sap SH2 domain. The closely related Eat2 SH2 domain, however, exhibited much less stringent binding specificity to PAK2. A degenerate phosphopeptide library experiment indicated that Sap and Eat-2 bind the T-I-Y-X-X-(V/I) motif (Poy et al., 1999). Though the direct interaction between PAK2 and Sap has not been shown experimentally before, a computational method using binding free energy estimation and peptide sequence analysis predicted that PAK2 Y194 was a potential Sap SH2 binding site (McLaughlin et al., 2006). The binding of Sap to Fyn has been previously described (Chan et al., 2003; Latour et al., 2003), and Sap has been shown to enhance Fyn-induced SLAM phosphorylation (Sayos et al., 1998). These results combined with our data suggest a possible role of Sap in Fyn-mediated PAK2 superactivation.

Forward-phase methods, in which the SH2 domains are coupled to a solid support and the analyte is applied in the soluble phase, have been used for SH2 profiling (Jones et al., 2006). A crucial difference between these forward-phase methods and the reverse-phase Rosette assay presented in this study is the binding kinetics of these two methods. Forward-phase methods are hindered by poor binding kinetics due to the small amount of phosphoproteins in the sample. Thus, these approaches work best when the purified analytes, such as phosphopeptides, are used in high concentrations enabling efficient analyte binding to the immobilized SH2 domains. Additionally, the coupling of SH2 domains to the solid support may destroy SH2 domain binding activities. However, SH2 domains are rather stable and have been successfully used in (forward-phase) protein arrays (Jones et al., 2006), suggesting that these are minor obstacles. Reverse-phase assays, however, are better for complex samples, such as cell lysates, which contain relatively small amounts of phosphoproteins. Because SH2 domains generally recognize short peptide sequences, immobilization of the phosphoproteins is unlikely to abrogate the recognition by SH2 domains (Machida et al., 2003).

The specificity of SH2 domains enable them to be used as tools for profiling the global tyrosine phosphorylation pattern of cells and tissues (Dierck et al., 2006;

Dierck et al., 2006; Machida et al., 2003; Machida and Mayer, 2005). Global SH2 binding patterns provide insights into the signaling output of a tyrosine kinase pathway. The global SH2 binding profile can provide information regarding the activity of the signaling pathway and can provide clues as to which interactions are likely to occur *in vivo*. The changes in the overall concentration of SH2 binding sites, and thus the SH2 binding profile *per se*, provide information as to which interactions will change most notably under various conditions. We examined SH2 binding patterns in fibroblasts cultured in adherent and suspended states and found that a notable portion (25%) of the tested SH2 domains exhibited increased binding in adherent states, indicating that the increased numbers of SH2 binding sites are present under adherent conditions. Paxillin, was selected as an adhesion-dependent protein for the SH2 profiling studies, and it was found to interact with the Csk SH2 domain. This interaction also has been described by others (Sabe et al., 1994). The use of SH2 profiling allowed us to observe notable ligand-binding specificity of Csk, and our analysis revealed that the interaction of Csk SH2 domain to paxillin was dependent on adhesion.

Tyrosine kinases regulate cell proliferation and survival and are important factors for the development of cancer (Blume-Jensen and Hunter, 2001; Krause and Van Etten, 2005). Thus, profiling the global state of tyrosine phosphorylation of a cell and identifying activated signaling pathways are important for understanding many physiological and aberrant functions of cellular signaling. A quantitative assessment of the SH2 binding pattern could be used as a diagnostic tool in clinical applications to classify a tumor sample for prognosis and prediction (Dierck et al., 2006; Nollau and Mayer, 2001; Yaoi et al., 2006). Activating mutations in the receptor tyrosine kinases are known to stabilize their activity and promote sustained downstream signaling (Kong-Beltran et al., 2006).

A recent study performed SH2 profiling on lung cancer samples, and they found that the Ras signaling pathway was particularly strongly activated downstream of the mutated EGF receptor (Machida et al., 2010). In this study, the SH2 binding profile correlated with abnormally activated EGF receptor-mediated downstream signaling. Additionally, the SH2 binding profile could be used as a biomarker for predicting sensitivity of cancer cells to tyrosine kinase inhibitor treatment. This study suggests that SH2 profiling could be useful for identifying key therapeutic

targets for cancer treatment because it reveals specific characteristics of different of cancers types.

Our data demonstrate that the majority of SH2 domains bind to their ligands in a pTyr-dependent manner, which suggests that SH2 domains are more devoted pTyr-binding modules than has been anticipated. Moreover, our data show that some SH2-binding sites were capable of binding multiple SH2 domains, suggesting that SH2 domains can share binding specificities. In a global comparison, only some of the closely related SH2 protein families exhibited similar binding patterns, suggesting that the SH2 ligand-binding site *per se* is relevant for SH2/ligand binding. We also presented a method to observe the binding of SH2 domains to cellular proteins by exploiting a high-throughput approach based on large-scale analysis and reverse-phase protein arrays. Our data show that SH2 domains differ from each other in number of their ligands in the cell, indicating different roles for SH2-binding phosphoproteins in cellular signaling networks. Our SH2-profiling method can quantify SH2 binding sites with high sensitivity; therefore it is a potential tool for deciphering tyrosine kinase pathway. However, if identification of specific SH2-binding partners is required, additional methods are needed.

9.2 Nef, PAK2 and ADAM15 preferred SH3-interactions by phage display

Modular protein binding domains mediate complex protein interaction networks during normal and pathological conditions within the cell. The SH3 domain is one of the most common domains found in eukaryotic genomes. We generated a virtually complete collection of human SH3 domain sequences and used phage display as a tool to identify SH3 domains that bound strongly to our target proteins (Nef, PAK2 and ADAM15). The SH3/ligand interactions identified by phage screening were further confirmed and characterized using a robust and semi-quantitative binding assay. Despite the different design and function of our assay, the measured affinity values correlated well with the numerical values that were obtained previously by other methods (e.g., surface plasmon resonance (Lee et al., 1995; Manser et al., 1998)), supporting the validity of our assay. Notably, the

measured binding affinities obtained from our assay were in agreement with the frequency of specific clones found in the phage display screen.

SH3 domains have been described to recognize short linear peptides. However, the protein interaction network study by Landgraf and colleagues tested the binding of 1500 short peptides for eight SH3 domains, and they observed K_D values that were generally higher than 10 µM (Landgraf et al., 2004). The lack of high-affinity SH3/ligand binding in the nanomolar range in their study might be attributed to the failure of short peptides to correspond to their native protein. Many examples indicate complex ligand recognition modes by SH3 domains; thus we used full-length proteins (Nef) or well-defined complete domains (PAK2 and ADAM15) as ligands in our study.

The phage display screen using human SH3 domain proteome confirmed several previously reported Nef, PAK2 and ADAM15 SH3-interactions, but the screen also identified new and potentially important SH3 domain-containing interaction partners for PAK2 and ADAM15. Because Hck is expressed in macrophages but not in T cells, thus, the crucial SH3-binding partner for Nef in T lymphocytes has remained unresolved. Despite the failure to identify other potential SH3-ligands for Nef that are expressed in T cells, our studies confirmed that Hck is the preferred SH3-binding partner for Nef. Moreover, these studies may indicate that a Hck-like high-affinity ligand for Nef may not exist in T cells. In addition, the SH3-mediated Nef-interactions in T cells may be different and not require such high-affinity binding. The established binding affinities for Nef with Fyn-SH3 (15.8 µM), Src-SH3 (14.3 µM) and Lck-SH3 (10.6 µM) (Arold et al., 1998) indicate weak interactions, but these affinity values are not uncommon among SH3/ligand complexes. We failed to select the Fyn, Src and Lck SH3 domains using Nef in the phage display screen, suggesting that relatively strong affinity values in the low micromolar range are necessary for the positive selection of specific SH3-phages in this system. It has also been postulated that these Src family kinases and the Nef protein may exist in the same multiprotein complexes in T cells without directly binding to each other (Saksela, 2011). Thus, it is possible that Nef-interacting SH3 proteins (other than the high affinity Nef-binding partners Hck and Lyn) cannot be determined by affinity-based methods but that functional assays should be used (Saksela, 2011).

PAK2 also selected known high-affinity SH3 partners, such as the PIX family proteins. PIX family SH3 domains were identified as the preferred PAK2 binding partners of PAK2. The binding of PIX to PAK regulates the activation and localization of PAK, and the PIX/PAK-interaction exhibits an exceptionally high binding affinity (K_D value of 24 nM) (Manser et al., 1998). Similarly, the role of Nck in recruiting PAK1 proteins to the membrane has been documented (Galisteo et al., 1996; Zhao et al., 2000). PAK2 also selected the third SH3 domains of vinexin-ArgBP2-ponsin family members in our phage display screen. ArgBP2 promotes cell survival and binds to both Akt and PAK1 kinases (Yuan et al., 2005). In addition, we found a novel PAK2-interacting SH3 domain of a protein dubbed POSH2; POSH2 is homologous to POSH1 (Tapon et al., 1998).

SH3 domains of the novel SNX33 protein and of nephrocystin were identified as preferred binding partners of ADAM15. A protein interaction assay demonstrated that sortin nexin SNX33 bound to ADAM15 with higher affinity than SNX9, which was previously shown to bind ADAM15 (Howard et al., 1999). Subsequent studies have confirmed the ADAM15/SNX33 interaction *in vivo* (Kleino et al., 2009). The related ADAM15-interacting SNX9 protein modulates membrane dynamics, and thus it has been suggested that SNX33 participates similarly in protein sorting (Lundmark and Carlsson, 2009). Fish is composed of a similar combination of PX and SH3 domains and was previously found to interact with ADAM15 (Abram et al., 2003). In addition, N-Src was found to bind to ADAM15, which has been described to interact with other homologous Src kinases (Poghosyan et al., 2002). Nephrocystin has not been shown to bind ADAM family proteins but identifying its SH3 domain as one of the two preferred partners of ADAM15 is of particular interest because this protein is defective in hereditary kidney disease (Hildebrandt et al., 1997). Interestingly, this disease is characterized by defects in cell adhesion (Hildebrandt and Otto, 2000), and ADAM proteins play a role in this process. Nephrocystin binds via its SH3 domains to proline-rich motifs in proteins that are involved in focal adhesion signaling complexes (Hildebrandt and Otto, 2000). Like nephrocystin, ADAM15 localizes to cell-cell contact sites (Ham et al., 2002) and it could be speculated that the ADAM15/nephrocystin complex plays a role in cell adhesion.

In addition to the novel interactions found in our study, the data suggest that high-affinity interactions are more common among SH3 domains than was

82

previously assumed. Moreover, our results implicate that certain SH3-domain/ligand interactions are very specific, reflecting the importance of SH3 domain-mediated binding events for determining ligand binding. The SH3 phage library system revealed high-affinity and selective SH3-interactions, making it a valuable tool for revealing SH3-mediated cellular networks. However, traditional approaches are still needed to confirm the importance of the interactions.

## 9.3 The novel POSH2 protein and its role in JNK activation

Apoptosis is a crucial event in the development of an organism, and its regulation is important for controlling cell proliferation. Controlled cell death is also important for responding to cell damage and infection. JNK signaling has been reported to play a pivotal role in apoptosis, and scaffold proteins have been implicated in enhancing the efficiency and specificity of the JNK signaling module (Dhanasekaran and Reddy, 2008).

Previously reported POSH scaffold proteins play a clear role in JNK signaling by facilitating interactions between molecular partners in the signaling complex. In addition to the previously reported POSH proteins (POSH1 and POSH3), we have described a new member of the POSH family, POSH2. Like its family members, POSH2 contains multiple SH3 domains that are potentially involved in interactions with many PxxP-motif-containing proteins (see Figure 14 and Appendix 2). In our phage display screen, we found that the third POSH2 SH3 domain interacted with N-terminal PAK2 (in communication **II**). However, our attempts to further analyze the PAK2/POSH2 interaction with full-length proteins failed, despite the clear interaction detected between their complete domains. We hypothesize that PAK2 and POSH2 play a role in different functional contexts, which may explain why full-length proteins do not interact with each other.

We compared POSH2 sequences to other POSH proteins and we found that POSH2 contains a unique N-terminal extension, which is highly conserved among different species. We found no evidence of a localization signal in the POSH2 sequence, and thus the role of this unique N-terminus remains unsolved.

Similar to POSH1 and 3, our results implied that the POSH2 RING domain possesses E3 ubiquitin ligase activity. Many E3 ligases are known to be self-ubiquitinated and are then targeted for degradation. Our findings indicated that POSH2 self-ubiquitination was associated with decreased cell survival and POSH2 protein expression, supporting the idea of an autoregulatory mechanism for controlling POSH protein levels and stability by protein degradation (Wang et al., 2010; Wilhelm et al., 2007a; Xu et al., 2003). Previous reports on POSH proteins have demonstrated similar effect of the RING domain to the stability of the POSH proteins (Tsuda et al., 2005; Wilhelm et al., 2007a; Xu et al., 2003). Unexpectedly, we did not observe a consistent POSH stabilizing effect of the disrupted RING domain when POSH2 was compared to the wild-type protein. The use of the RING-mutated POSH2 construct in the co-transfection assay slightly increased the transfection efficiency/cell survival. These results presented in communication **III** do not necessarily conflict with the findings of the two other POSH family members. However, differences in protein stability may be explained by methodological differences and/or the use of different cell lines.

Rac1 GTPase has been shown to regulate JNK signaling (Teramoto et al., 1996). In response to apoptotic stimuli, Rac becomes active, GTP-loaded and can interact with downstream effectors in the JNK signaling pathway. POSH1 was initially identified as a Rac-binding protein (Tapon et al., 1998). This interaction was found to be specific to Rac1 and was thought to be mediated by a unique Rac-binding domain (RBD) (Tapon et al., 1998). Classically, Rac and Cdc42 GTPases bind a core sequence called the CRIB domain (I-S-X-P-(X)$_{2-4}$-F-X-H-X-X-H-V-G), in their target proteins (Burbelo et al., 1995). We performed sequence alignments of POSH proteins with other Cdc42 and Rac1-binding proteins, and we found a conserved partial CRIB domain (I-S-X-P) in the RBD. A similar "semi-CRIB" motif, which contained a slightly larger portion of the classical CRIB domain (I-S-X-P-X-X-F), has been reported in the Par6 partition-defective protein (Garrard et al., 2003). The Par6, semi-CRIB domain is essential for binding Cdc42 and the presence of the adjacent PSD95/Discs Large/ZO-1 (PDZ) domain is also required (Garrard et al., 2003). The PDZ domain stabilizes CRIB domain structure in Par6. This PDZ domain is absent in POSH proteins but the involvement of another stabilizing structure for the partial CRIB-domain is possible.
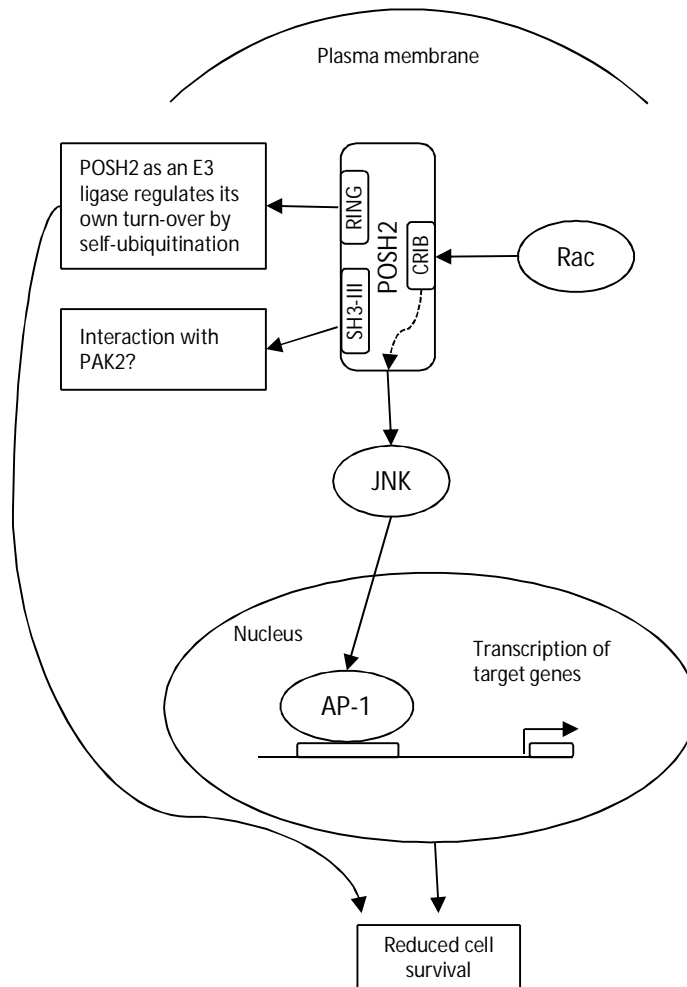
Figure 14. Overview of the studied POSH2 functions and suggested roles in signaling pathways. GTP-bound and activated Rac1 binds to the POSH2 partial Cdc42/Rac interactive binding domain (CRIB), which is required for Rac1 binding. This interaction is suggested to activate JNK kinase, which translocates into the nucleus and controls gene expression by regulating the activity of transcription factors. This leads to the formation of activating protein-1 (AP-1) and to increased expression of target genes (e.g. pro-apoptotic genes), which leads to reduced cell survival. The POSH2 functions as an E3 ligase, which can control its own turn-over by self-ubiquitination. POSH2 contains multiple SH3 domains and mediates PAK2 binding via its third SH3 domain. However, an interaction between full-length POSH2 and PAK2 could not be detected, thereby setting a stage for future studies.

Utilizing POSH2 CRIB variants (POSH2-ISP (I74N, S75P and P77A), POSH2-I403N and POSH2-S404P), we demonstrated that the binding of active Rac1 to POSH2 is dependent on an intact POSH2 I-S-X-P motif. We also showed that the full-length POSH2 protein carrying a mutation in the partial CRIB domain (S404P)

impaired the activation of AP-1/JNK. Together, these results suggest that POSH2 activates JNK signaling. An intact partial CRIB domain, which is necessary for POSH2/Rac binding, is crucial for the activation of JNK signaling. We hypothesize that the partial CRIB domain is functionally similar to the classical CRIB domain of PAK2, which binds to p21 GTPase via this domain (Zhao et al., 1998).

In conclusion, the results presented in communication **III** suggest that the novel POSH2 protein shares structural and functional similarities with the two previously reported POSH proteins. We hypothesize that POSH2 plays a scaffolding role in the apoptotic JNK signaling pathway. Our data provide a novel perspective to the Rac-binding motif in POSH2, which we implicate to contain a partial CRIB domain. We show the importance of the partial POSH2 CRIB domain for binding p21 GTPases *in vitro*, though additional stabilizing and scaffolding structures for the POSH2 CRIB domain may exist. Our results demonstrate the importance of the partial POSH2 CRIB domain for binding to Rac1 GTPase and further implicate that this interaction promotes JNK activation *in vivo*.

10. CONCLUSIONS

Modular protein domains mediate protein-protein interactions that are important for transmitting information within a cell and thus enable the cell to maintain homeostasis and to respond to changing conditions. SH2 and SH3 domains both constitute large families of conserved protein interaction domains, which are critical for many cellular processes ranging from apoptosis to cell survival. Given the importance of these domains in the global proteome, it is necessary to develop methods for identifying specific proteins in selected cellular signaling cascades and to characterize their interaction profiles for research applications and diagnostic use.

Tyrosine phosphorylation creates binding sites for SH2 domains, which mediate downstream signaling that leads to various cellular responses. In this study, the SH2 domains were demonstrated to be preferred pTyr-binding domains, suggesting the unique nature of these protein-binding modules and supporting the idea of using these domains to characterize the pTyr status of a cell. SH2 domains exhibited different protein binding preferences, which reflected the characteristics of specific SH2 domain-containing proteins that recognize different ranges of cellular proteins. The quantitative reverse-phase Rosette assay combined with a far-Western approach provided sufficient sensitivity for detecting normal levels of tyrosine phosphorylation in unpurifed samples, and thus this approach is applicable for high-throughput profiling.

In this work, we have determined the SH3 domains in the human genome and have investigated the binding of SH3 domains to Nef, ADAM15 and PAK2. The phage-display method provided an unbiased approach for identifying SH3 domains that bind the target proteins with high affinity. The competitive nature of the system enabled the screening results to be dictated by binding affinity because other factors influencing the selection results are minimal. Therefore, this method provides more reliable results when compared to experimental approaches such as yeast-two hybrid screening. Despite the character of modular protein domains to recognize short linear peptides, more complex SH3/ligand recognition modes have also been

described, for example among binding events of high affinity (e.g., Nef/Hck SH3). Therefore, the use of full-length proteins or well-defined domains is expected to provide more complete characterization of important SH3/ligand interactions. Although high affinity binding *per se* does not guarantee that the SH3/ligand complex is important, it is unlikely that specific and strong interactions would have evolved by chance. SH3 domains are generally considered to bind to ligands with low affinity; however, we identified many SH3-mediated interactions with ADAM15 and PAK2 that exhibited nanomolar affinities, suggesting a more important role for SH3 domains in cellular signaling networks. However, further experiments are still needed to confirm the significance of these novel interactions in the cellular context.

A novel PAK2-interacting SH3 domain was found to be part of a larger gene product, which we named POSH2 due to its close similarity to a previously reported scaffold protein called POSH1. The multidomain character of POSH2 suggests that it can mediate multiple SH3/ligand and partial CRIB/Rac1 interactions, implying that the protein may function as a scaffold protein. The presence of a RING domain enables POSH2 to function as an E3 ligase and provides a mechanism for POSH2 to regulate the turnover of cellular proteins. POSH2 was shown to promote JNK-mediated apoptosis, elucidating a new protein member to regulate cell death.

Our studies indicated that the global SH2 binding pattern extended to the whole proteome provides information regarding the activity of signaling pathways, and the likely interactions occurring *in vivo* and the changes in concentration of SH2 domain ligands under various cellular conditions. Tyrosine phosphorylation has an important role also in unregulated cellular functions in instances such as cancer; therefore, SH2 profiling could be used as a molecular diagnostic tool for characterizing and classifying tumor samples. The phage display-based approach exhibited high selectivity and affinity for discovering SH3/ligand interactions and should therefore be a valuable tool for investigating cellular SH3-mediated signaling networks. The characterization of the novel SH3 protein suggested a multifunctional role for POSH2, thereby setting the stage for future detailed functional studies.

11. ACKNOWLEDGEMENTS

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13. SUPPLEMENTARY DATA

Appendix 1. The POSH2 N-terminal extension sequence does not contain a targeting sequence. U2OS cells were transfected with either POSH2 (aa 1-70)-GFP (ATG1+2), POSH2 (aa 46-70)-GFP (ATG2) or plain GFP. Cells were stained with phalloidin to visualize the cytoskeleton. Transfected GFP and Alexa Fluor 568 Phalloidin were visualized by fluorescence microscopy. *(Reprinted from the original communication **III** (Supplementary Figure B); with permission from Elsevier).*

| ATG1+2 | ATG2 | control |
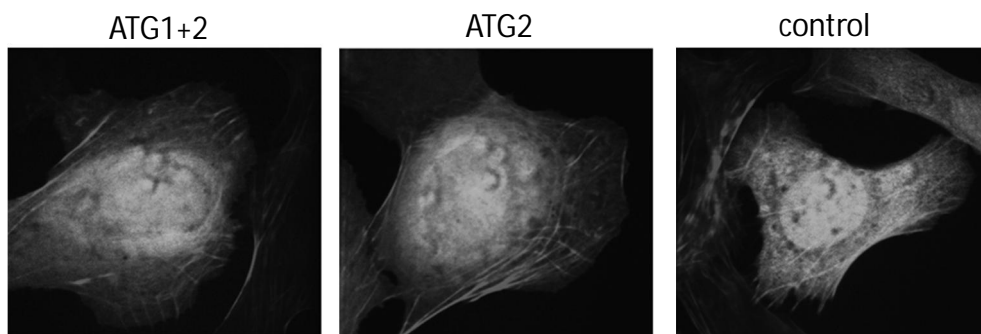
Appendix 2. Alignment of POSH proteins. The human POSH2 amino acid sequence is aligned with different POSH proteins from human (H), rat (R), and mouse (M). SH3 domains are indicated with brackets, and the Rac-binding domain (RBD) is highlighted with dashed brackets. The I-S-X-P amino acids involved in Rac1 binding are indicated with filled circles, and the L385 and P414 mutations (generated as control mutations) are shown with open circles. Amino acids constituting the RING finger domain are highlighted with asterisks as the sites mutated in this domain (with bigger asterisks). *(Reprinted from the original communication **III** (Supplementary Figure A); with permission from Elsevier).*

14. ORIGINAL COMMUNICATIONS

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Resource

High-Throughput Phosphotyrosine Profiling Using SH2 Domains

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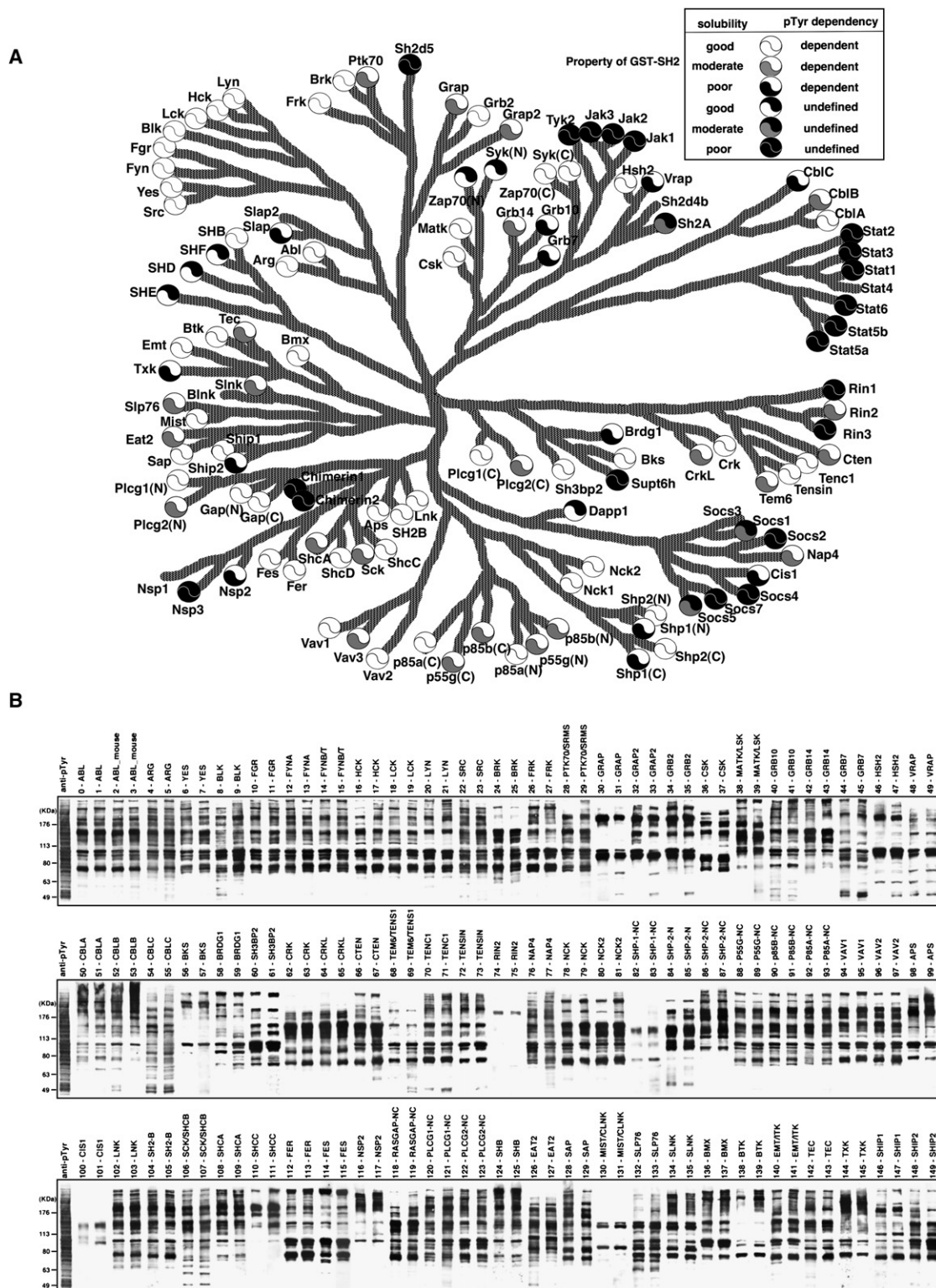DOI 10.1016/j.molcel.2007.05.031

SUMMARY

Protein tyrosine phosphorylation controls many
aspects of signaling in multicellular organisms.
One of the major consequences of tyrosine
phosphorylation is the creation of binding sites
for proteins containing Src homology 2 (SH2)
domains. To profile the global tyrosine phos-
phorylation state of the cell, we have developed
proteomic binding assays encompassing
nearly the full complement of human SH2
domains. Here we provide a global view of
SH2 domain binding to cellular proteins based
on large-scale far-western analyses. We also
use reverse-phase protein arrays to generate
comprehensive, quantitative SH2 binding
profiles for phosphopeptides, recombinant
proteins, and entire proteomes. As an example,
we profiled the adhesion-dependent SH2 bind-
ing interactions in fibroblasts and identified
specific focal adhesion complex proteins
whose tyrosine phosphorylation and binding
to SH2 domains are modulated by adhesion.
These results demonstrate that high-through-
put comprehensive SH2 profiling provides valu-
able mechanistic insights into tyrosine kinase
signaling pathways.

INTRODUCTION

Protein tyrosine kinases (PTKs), protein tyrosine phospha-
tases (PTPs), and their substrates play a critical role in

regulating processes such as proliferation, differentiation,
motility, and immune responses, as well as pathological
conditions such as cancer (Hunter, 2000). Profiling the
global tyrosine phosphorylation state of cells under vari-
ous physiological conditions has therefore become an
important goal. Current efforts in this area fall into two
broad categories, identification based and detection
based (Machida et al., 2003). The first relies on sensitive
mass spectrometry (MS) for unambiguous identifications
of phosphopeptides and usually requires phosphoprotein
enrichment and large amounts of sample (Kim et al., 2005;
Kumar et al., 2007; Olsen et al., 2006). The latter approach
generally exploits the potential of antibodies that recog-
nize specific phosphorylated sites, or pan-anti-phospho-
tyrosine (anti-pTyr) antibodies. Although detection-based
methods may not result in phosphoprotein identification,
they have advantages in terms of sensitivity and through-
put. For example, reverse-phase protein microarrays cou-
pled with phosphospecific antibodies have been used to
profile immunological responses and cancer progression
(Chan et al., 2004; Paweletz et al., 2001). However, phos-
phospecific antibodies appropriate for quantitative analy-
sis are available for relatively few phosphorylated sites;
thus comprehensive profiling of tyrosine phosphorylation
is still a challenge.

A primary mechanism used by the cell to interpret phos-
photyrosine-mediated signals relies on small, modular
protein domains that bind specifically to tyrosine-phos-
phorylated proteins. The Src homology 2 (SH2) domain
is the most prevalent of these modules and plays a central
role in tyrosine kinase signaling pathways (Machida and
Mayer, 2005; Pawson et al., 2001; Sadowski et al., 1986;
Yaffe, 2002). Recently, Nash and coworkers reported
that the human genome contains a total of 120 SH2
domains in 110 distinct proteins (Liu et al., 2006). The

Figure 1. Characterization of Purified SH2 Domains

(A) Solubility and pTyr dependency of GST-SH2 fusion proteins. GST-tagged SH2 domains were expressed in *E. coli*, and solubility was classified as good (>50%), moderate (25%–50%), or poor (<25%) based on anti-GST immunoblotting of soluble fraction relative to whole bacteria lysate. pTyr dependency is assessed by pull-down assay and far-western blotting (for details, see Table S1). Dendrogram is adapted from Liu et al. (2006).

SH2 domain is relatively small (~100 amino acids) and can fold independently; hence the isolated domain can be expressed in bacteria, purified, and used for in vitro binding assays (Mayer et al., 1991).

SH2 domains differ in their binding preferences for specific phosphorylated ligands, resulting in specificity in signal transduction (Pawson, 2004). The ligand selectivity of SH2 domains has been investigated in vitro using directed phosphopeptide library screening (Songyang et al., 1993). These studies revealed that, in addition to phosphotyrosine, amino acids in positions from −2 to +4 relative to phosphotyrosine contribute to high-affinity binding in most cases; more extended contacts (−6 to +6) have been observed in some structural studies (Hu and Hubbard, 2005; Liu et al., 2006; Pascal et al., 1994). Although SH2 domains have been studied extensively both biochemically and structurally, numerous questions remain to be addressed. For example, do all SH2 domains bind phosphotyrosine? Do specific tyrosine-phosphorylated sites bind to one or many SH2 domains? Do SH2 domains in closely related protein families have similar binding specificity? To what extent do SH2 domains vary in the number of binding partners in the cell? To answer such questions, it is clear that comprehensive experimental data are needed to complement computational approaches (Joughin et al., 2005).

We first proposed using SH2 domains as the basis for a robust, simple, and biologically relevant method to profile the global tyrosine phosphorylation state (Machida et al., 2003; Nollau and Mayer, 2001). Because SH2 domains bind selectively to specific tyrosine-phosphorylated sites and play an important role in mediating tyrosine kinase signaling in vivo, we reasoned that a battery of SH2 domains could be used to probe differences in global patterns of tyrosine phosphorylation relevant to signaling. We therefore established a modified far-western method using glutathione S-transferase (GST)-SH2 fusion proteins (Nollau and Mayer, 2001). This approach, which we term SH2 profiling, holds promise as a means of characterizing changes in tyrosine phosphorylation in response to physiological signals on a system-wide level, and also as a molecular diagnostic tool for classifying clinical samples such as tumors (Dierck et al., 2006; Machida et al., 2003).

In the present study, we employ a comprehensive, quantitative, high-throughput phosphotyrosine profiling strategy incorporating nearly the entire complement of human SH2 domains. First, we have generated GST-SH2 fusions for currently known human SH2 domains. For the 74 SH2 domain probes with sufficient solubility, we compare their binding preferences for cellular proteins by far-western blotting and evaluate the correlation between similarity in binding patterns and sequence similarity (evolutionary distance). Next, we have developed a high-throughput quantitative reverse-phase SH2 bind-ing assay, dubbed the SH2 Rosette assay, which allows a large number of binding reactions to be performed simultaneously with high sensitivity and reproducibility. In a two-step screening strategy, small amounts of protein sample (whole-cell lysate) are used for initial Rosette screening, followed by gel electrophoresis-based far-western analysis with selected SH2 domains to confirm screening results and set the stage for identification of specific SH2 binding proteins of interest. This flexible SH2 profiling approach provides an effective tool for analyzing the tyrosine phosphoproteome and complements existing proteomic strategies.

RESULTS

Characterization of SH2 Domains

A global SH2 profiling method should incorporate a comprehensive set of SH2 domain probes. We collected cDNAs for all presently known SH2 domains and constructed bacterial vectors expressing N-terminally GST-tagged SH2 proteins. SH2 domains are highly conserved in their overall structure; thus the domain boundaries were readily discerned from primary sequence. To date we have generated 110 SH2 domain constructs, representing 95% (114/120) of all human SH2 domains (104/110 of SH2 domain-containing proteins) (Liu et al., 2006). We categorized the solubility of GST-SH2 domains into three classes (good, moderate, and poor) based on small-scale protein purification experiments (Figure 1A and see Table S1 and Table S2 in the Supplemental Data available with this article online). Approximately half of the GST-SH2 domains tested were highly soluble and well behaved when expressed in *E. coli*, but the remainder were less soluble. Poor solubility was particularly associated with several families of related SH2 domains, as illustrated in the dendrogram (Figure 1A). SH2 domains of the Stat, Socs, and Jak protein families were particularly insoluble, as also noted by others (Babon et al., 2006; Jones et al., 2006). Jones et al. reported some success solubilizing such domains by denaturation and refolding, but we did not try this approach.

Next we determined the phosphotyrosine dependency of SH2 domain binding. This was evaluated by (1) GST-SH2 pull-down assays of lysates from pervanadate-treated or untreated cells, followed by anti-pTyr immunoblotting (pervanadate inhibits endogenous tyrosine phosphatases, thus strongly enhancing tyrosine phosphorylation in vivo); (2) GST-SH2 pull-down assays of pervanadate-treated lysates in the presence/absence of phenyl phosphate, followed by anti-pTyr immunoblotting (phenyl phosphate specifically competes with phospho-tyrosine for SH2 domain binding); or (3) far-western blotting of pervanadate-treated/untreated cell lysates (Figures 1A and 1B and Figure S1). Based on these assays, 76%

(B) Binding preferences of SH2 domains. Pervanadate-treated cell lysate (mixture of NIH 3T3, A431, HepG2-PDGFR, and MR20) was separated on gradient gels and identical blots probed with GST-SH2 domain probes as indicated. Binding conditions and exposure times were optimized to provide approximately equivalent total signal for each domain. Results from two independent experiments are shown.

(85/112) of GST-SH2 probes tested showed clear pTyr-dependent binding (Table S1), including the Rin2 SH2, which contains histidine at the βB5 position instead of the highly conserved arginine in FLVRES motif believed to be critical for phosphotyrosine binding (Figure S1B; Kuriyan and Cowburn, 1997; Liu et al., 2006; Machida and Mayer, 2005). The remaining probes were highly insoluble and did not display any detectable binding activity, therefore phosphotyrosine dependency could not be evaluated (indicated as "undefined" in Figure 1A). We note that some of these poorly soluble SH2 domains, e.g., Stat and Socs, have been shown to bind specific tyrosine-phosphorylated sites in cell-based assays. The fact that all evaluable GST-SH2s showed clear phosphotyrosine-dependent ligand binding confirms that the SH2 domain is a dedicated phosphotyrosine binding module, in contrast to other protein binding modules such as the PTB domain, where most examples bind in a phosphorylation-independent fashion (Uhlik et al., 2005).

The protein binding specificity of the 74 human SH2 probes with adequate solubility and low nonspecific binding was investigated by pull-down and far-western blotting experiments (Figure 1B, Figure S1, and Table S1). In both assays, SH2 domains displayed diverse binding patterns as expected. We noted that a few SH2 probes were positive in the pull-down assay (gave a strong signal when probed with anti-pTyr antibody) but gave relatively weak signals in the far-western assay. This may be due to the detection of indirect binding partners (i.e., other members of large pTyr-containing complexes) in the pull-down assay; by contrast, the far-western assay detects only direct binding partners.

To assess the similarity of the far-western binding patterns for different SH2 domain probes, the location and signal intensity of each band in the blots were quantified, and data were analyzed by a hierarchical clustering algorithm to provide a quantitative measure of overall similarity (Figure 2A). The reproducibility of binding patterns was very high, as demonstrated by coclustering of independent replicate samples for the same SH2 domain in almost every case (replicate was closest match in 70/74 cases). Coclustering of replicates also implies that, even for very closely related SH2 domains, subtle distinctions in binding specificity can be discerned by this approach. As might be expected, the SH2 domains from closely related protein families generally share similar, unique binding patterns; for example, binding patterns for Nck1 and Nck2, and for Grb2, Grap, and Grap2, are virtually identical (clusters 1 and 2, Figure 2A). Roughly a third of the SH2 domains, including those of almost all nonreceptor tyrosine kinases, clustered together in a group with relatively broad specificity (Figure 2A, cluster 3). However, more subtle distinctions within this cluster are clearly apparent. For example, the eight Src family kinases can be subclassified into two distinct families (Blk, Lyn, Hck, and Lck; and Fyn, Yes, Fgr, and Src) based on SH2 binding pattern (Figure 2A), which correspond to their classification based on sequence similarity (Figure 1A).

We also performed a global comparison of the relatedness of SH2 domains based on primary sequence (evolutionary distance) with relatedness based on binding patterns (Figure 2B). Overall, the correlation between sequence similarity and binding pattern was rather poor, except for closely related family members. This analysis also revealed several cases in which there was striking discordance between binding pattern and sequence similarity. For example, the SH2 binding patterns for the four closely related tensin family members (tensin, tenc1, Cten, and Tem6/Tens1) are quite distinct (see Figure 2B, blue). There are also a few examples of distantly related SH2 domains with very similar binding patterns, the most notable example being the Nap4 SH2 (a member of the SOCS family), which is very similar in binding pattern to Src family SH2 domains (Figure 2B, green). Agreement between clustering based on sequence similarity and binding pattern can also be visualized by comparing the two trees directly (Figure S2). In this analysis, SH2 domains for a number of small protein families such as the Cbls, Fer/Fes, and Sap/Eat2 show good agreement (Figure S2, red circles).
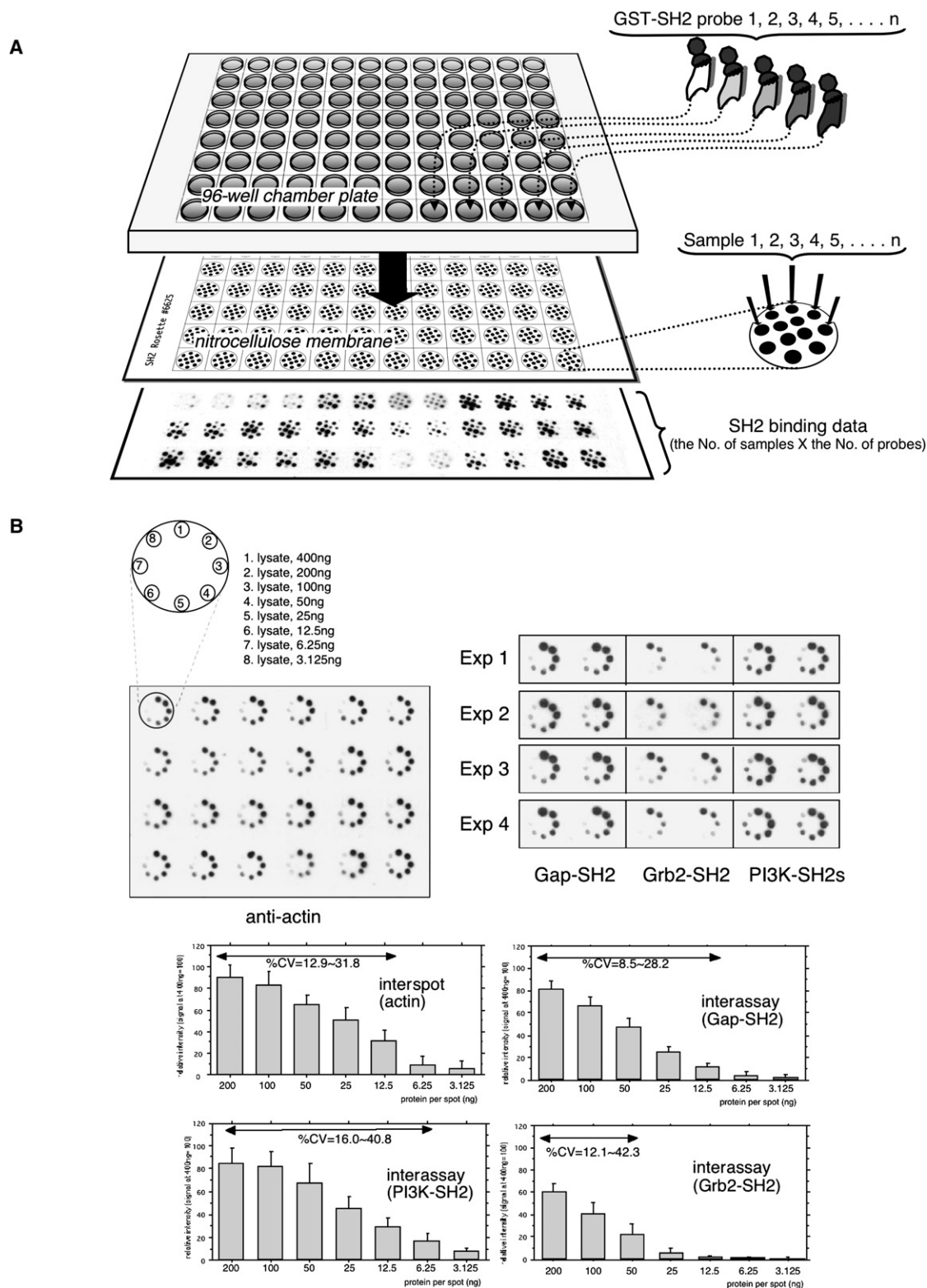
Development of High-Throughput SH2 Domain Binding Assay

We previously described the use of far-western blotting to profile the global phosphorylation state of a sample (Nollau and Mayer, 2001). This 1D gel electrophoresis-based approach requires relatively large amounts of protein, however, and quantitative analysis of binding patterns is difficult and highly sensitive to experimental variables such as electrophoresis conditions. In order to increase the throughput of the assay and decrease sample requirements without sacrificing data quality, we envisioned a two-step screening strategy, using quantitative reverse-phase dot blotting for initial profiling, followed by 1D gel analysis as needed. In reverse-phase assays, protein analytes are immobilized in the solid phase and probes such as antibodies or modular protein domains are applied in solution (Liotta et al., 2003; MacBeath, 2002). This approach has advantages where the concentration of analyte is low (e.g., tyrosine-phosphorylated proteins in whole-cell lysates), because efficient binding can be driven by high concentrations of the SH2 domain probe. By contrast, forward-phase assays (where domains or antibodies are arrayed and analyte is in solution) suffer from poor binding kinetics under these conditions, leading to relatively low signal and high background.

We have adopted a hybrid dot-blotting method, which we dubbed the SH2 Rosette assay, in which multiple samples (up to 12 or more) are manually spotted on a nitrocellulose filter in register with a well of a 96-well chamber plate. Duplicate sample arrays are spotted in multiple wells, allowing a maximum of 96 different SH2 domain probes to be tested in one experiment (Figure 3A). SH2 binding and washing are performed in the chamber plate, and binding is detected by chemiluminescence and

Figure 2. Bioinformatic Analysis of SH2 Domain Binding Patterns

(A) Unsupervised hierarchical clustering analysis of SH2 domain binding preferences. Each gel lane (Figure 1B) was divided into 48 bins, signal intensity of each bin was quantified by densitometry, and values were used as the basis for clustering.

(B) Pairwise distance values based on sequence alignment (Liu et al., 2006) (Y axis) plotted against pairwise (Euclidean) distance values based on binding patterns (X axis) for each pair of SH2 domains. Data for Src family kinases (SFKs) versus Src family kinases (red), Src family kinases versus Nap4 (green), and tensin family versus tensin family (blue) are highlighted.

quantified by densitometry. With this rapid and quantitative approach, it is possible to profile the level of binding to virtually all SH2 domains in the genome using minimal amounts of protein sample. A single spot consumes only 0.1 μl (<500 ng) of sample; thus no more than 50 μg sample is required for a 96-probe binding reaction. Accuracy of spotting and experimental variation was validated as shown in Figure 3B. Interspot variation (accuracy of spot-

ting) and interassay variation (experimental variation) were 12.9% and 8.5%–16.0%, respectively, for higher protein loading (50–200 ng per spot). As expected, slightly larger variation was observed closer to the limit of detection (<50 ng). Detection sensitivity varies depending on the specific SH2 domain and sample, but typically is in the range of 5–50 ng of whole-cell lysate per spot.

Figure 3. Strategy for High-Throughput SH2 Domain Binding Assay

(A) Overview of SH2 Rosette assay. Protein samples are immobilized on membrane in multiple duplicate arrays, and each SH2 probe is incubated with a set of protein spots in separate wells of a 96-well chamber plate. After ECL-based detection and densitometric quantitation, resulting values are used to compare different samples and SH2 probes.

SH2 Profiling for Specific Phosphopeptides

To decipher cellular signaling pathways, it is important to determine which SH2 domain(s) can bind with high affinity to specific tyrosine phosphorylation sites. We first tested the utility of the SH2 Rosette assay for unbiased screening both for phosphopeptides and for purified phospho-proteins.

Platelet-derived endothelial cell adhesion molecule (PECAM-1) is an immunoglobulin superfamily member expressed in endothelial cells, leukocytes, and platelets. It participates in cell signaling pathways via two potential phosphorylation sites in its cytoplasmic domain, tyrosines 663 and 686, both of which conform to the consensus for immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (Newman and Newman, 2003) (Figure 4, top). N-terminally biotinylated peptides corresponding to both sites were synthesized in both unphosphorylated (Tyr663 and Tyr686) and phosphorylated (pTyr663 and pTyr686) forms and immobilized on membranes.

As shown in Figure 4, SH2 domain probes differed in their ability to bind the two phosphorylated peptides, but in no case did they bind the unphosphorylated peptides. The binding results were consistent with previously reported interactions. Ten SH2 proteins (Shp1, Shp2, Src, Fyn, PLCγ1, SHIP, PI3K (P85α), Grb2, STAT3, and STAT5) have been reported to bind phosphorylated PECAM-1 (Newman and Newman, 2003), and the SH2 Rosette assay detected all but Grb2, which is thought to bind indirectly (Newman and Newman, 2003) (STAT probes were not used in our experiment due to their insolubility). We found that the N-terminal SH2 domain of Shp2 (Shp2N) preferentially interacted with pTyr663, while the tandem Shp2 SH2 domain probe (Shp2NC) bound both pTyr663 and pTyr686, consistent with a previous surface plasmon resonance (SPR) study (Pumphrey et al., 1999). We also uncovered previously unappreciated interactions, such as the strong binding of the Csk and Sap SH2 domains to pTyr686 (Figure 4). Thus this comprehensive approach can validate previously known interactions and identify new ones that can be further explored by hypothesis-driven experimentation.

We have previously reported that performing SH2 binding reactions under competitive conditions (in the presence of a single labeled SH2 probe and multiple unlabeled competitor SH2 domains) can improve binding specificity (Nollau and Mayer, 2001). To test the effects of competition in the Rosette assay format, we performed binding assays in the presence or absence of a cocktail of unlabeled SH2 domains as competitors. As shown in Figure S3, the overall binding patterns of SH2 domains to the PECAM peptides were comparable with or without competitors. However, competitive conditions did provide some additional information regarding those domains with the high-est specificity (relative affinity) for a site. For example, Shp2 SH2 probes bound well to pTyr663 even in the presence of excess unlabeled competing SH2 domains, suggesting this interaction is highly favored. On the other hand, binding of all SH2 domains tested to pTyr686 was dramatically reduced by competition, suggesting many SH2 domains bind this site with similar affinity.

SH2 Profiling of Recombinant Phosphoproteins

We also used the Rosette assay format to profile SH2 domain binding to purified recombinant proteins. Members of the p21-activated kinase (PAK) family are important regulators of cytoskeletal dynamics, cell motility, and cell survival (Bokoch, 2003). N-terminal tyrosine phosphorylation of PAK2 has been reported to lead to superactivation of the kinase, although the specific mechanism is not yet known (Renkema et al., 2002). The N-terminal half of PAK2 (amino acids 1–212, hereafter PAK2N; see Figure 5A), which contains three tyrosines, was tyrosine phosphorylated by expression in TKB1 bacteria, which harbor an activated EphB1 tyrosine kinase. To identify possible phosphorylation-dependent binding partners, purified PAK2 proteins were immobilized and subjected to SH2 Rosette analysis (Figure 5B).

Several SH2 domain probes bound strongly to PAK2N, including the Sap SH2 (arrow in bar graph, Figure 5B), although most bound poorly relative to the positive control (v-Abl-transformed cell lysate). To extend these findings, we carried out far-western analysis using wild-type PAK2N or mutants lacking specific phosphorylation sites (Y130F, Y139F, and/or Y194F), with SH2 probes from Sap and its close relative, Eat2 (Figure 5C). Binding of the Sap SH2 domain to PAK2N was completely abolished by the Y194F mutation, demonstrating that pTyr194 is critical for the interaction (Figure 5C, indicated by an arrow). On the other hand, no single site was essential for binding to the Eat2 SH2 domain. The differential dependence of Sap and Eat2 SH2 domains on pTyr194 was further confirmed by SPR analysis (Figure S4).

Sap, which consists of a single SH2 domain, is the product of the gene mutated in X-linked lymphoproliferative syndrome (XLP) (Sayos et al., 1998); its interaction with PAK kinases has not been reported. The binding consensus of Sap and Eat2 SH2 domains has been determined by random phosphopeptide library screening to be (T/S)IpYxx(V/I) (Poy et al., 1999). While sequences surrounding pTyr130 and pTyr139 do not strictly conform to this consensus (Figure 5A), pTyr194 matches reasonably well. The dramatic difference between Sap and Eat2 in their dependence on pTyr194 was not anticipated, however, and indicates that the Eat2 SH2 domain has a much less stringent binding specificity than that of its close relative Sap. Although the association of Sap and

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(B) Validation of SH2 Rosette assay. Various amounts of v-Abl-transformed 3T3 lysate were spotted in 24 duplicates on a nitrocellulose membrane and probed with anti-actin antibody to evaluate interspot variation (accuracy of spotting) (upper left), and three different GST-SH2 probes were used to evaluate interassay variation (upper right). Bar graphs show values of densitometric quantitation. %CV, coefficient of variation. Error bars indicate the SEM.
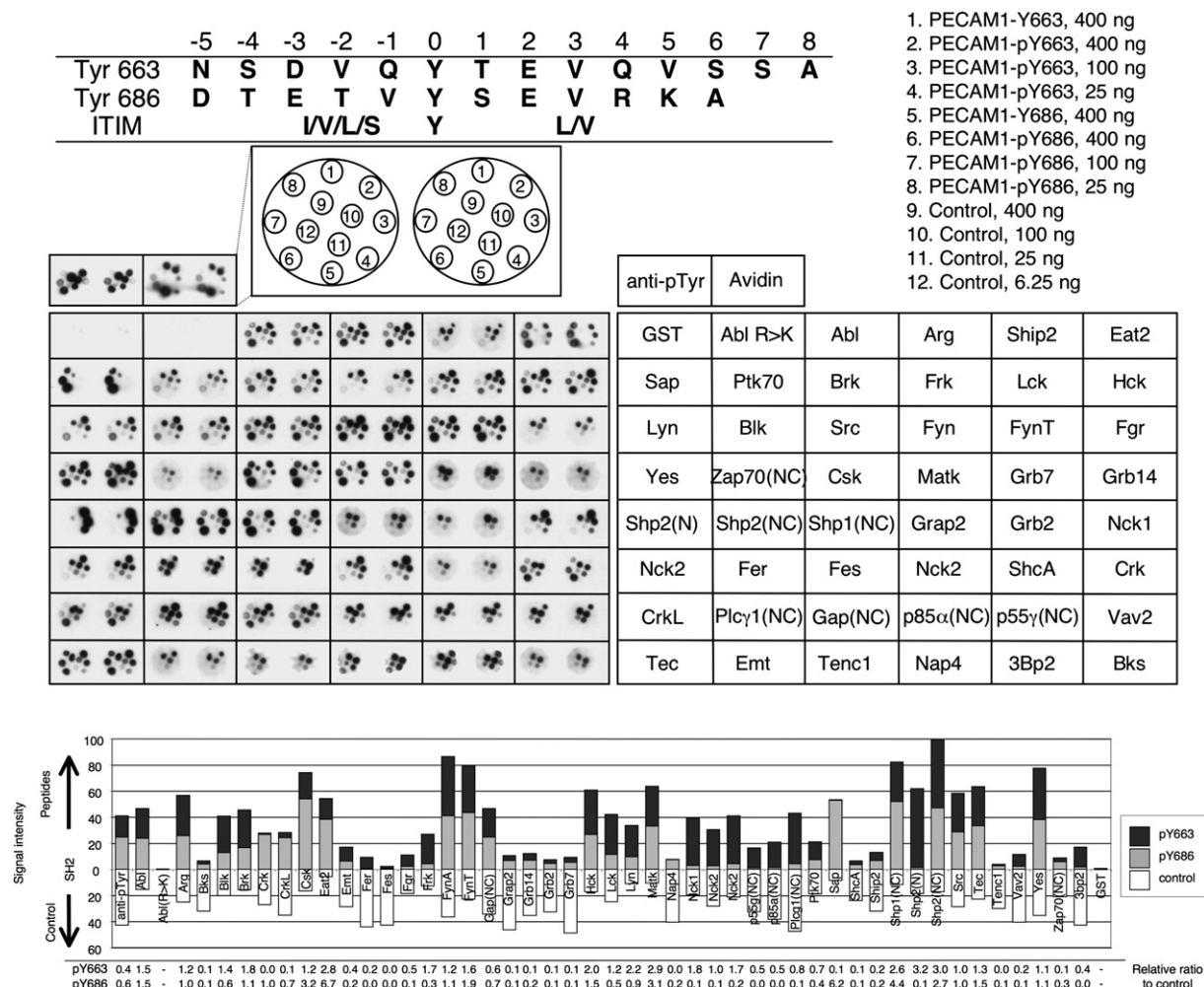
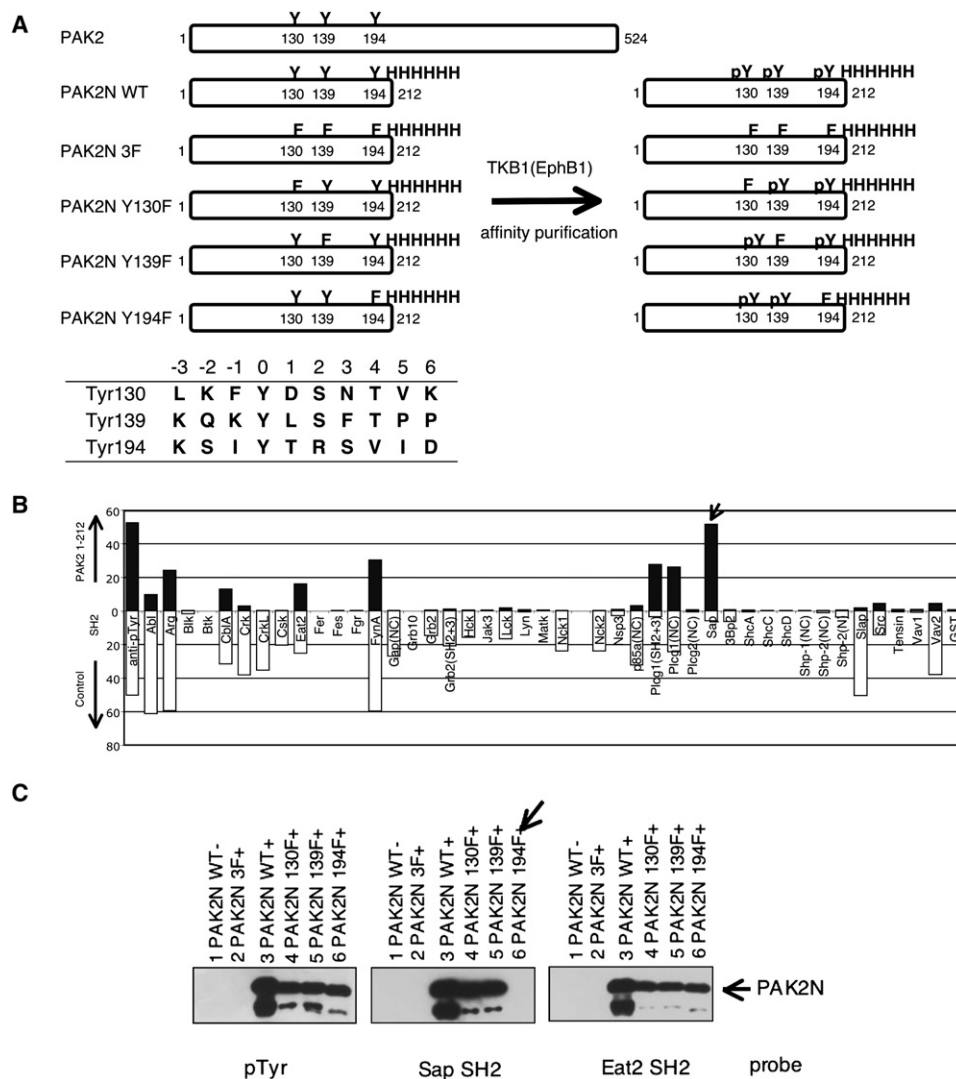**Figure 4. Profiling SH2 Binding to PECAM-1 ITIM Motifs**

Peptides corresponding to tyrosine 663 and 686 of human PECAM-1 were synthesized in phosphorylated (pY663 and pY686) or unmodified (Y663 and Y686) form. Indicated amount of peptides and internal positive control sample (pervanadate-treated v-Abl-expressing NIH 3T3 lysate) were immobilized as described in the Experimental Procedures. Blocked membrane was probed with anti-phosphotyrosine antibody (anti-pTyr), avidin-HRP (avidin), and GST-SH2 domains as indicated on the right, and signal levels for each binding reaction (summed values for multiple concentrations of peptide) were quantified (bar graph, bottom, arbitrary units). The "relative ratio to control" values indicate relative binding of each probe to phosphopeptides compared to positive control. For example, Shp2N probe showed strong preference for pTyr663 (3.2× more binding than control).

PAK had not before been experimentally documented, it is interesting to note that a bioinformatic approach using binding free energy estimation and peptide sequence analysis predicted the PAK2 Tyr194 site as a potential Sap SH2 binding site (McLaughlin et al., 2006).

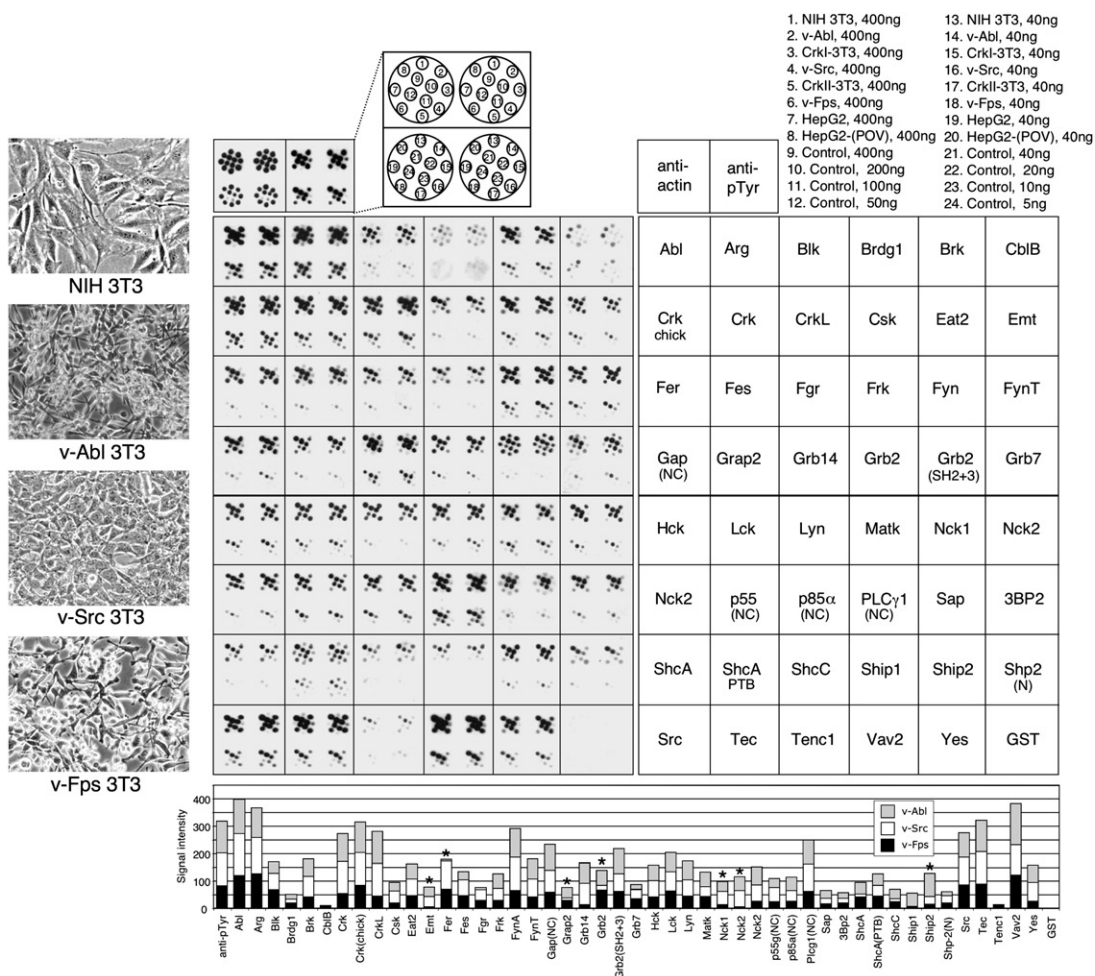**Application of Rosette Assay to Entire Proteomes**

We next tested whether the SH2 Rosette assay was sensitive enough to profile levels of SH2 binding sites in complex protein mixtures such a whole-cell lysates. We first analyzed lysates of cells transformed by the viral oncogene products v-Abl, v-Src, and v-Fps. Although all three of these activated tyrosine kinases can transform murine fibroblasts (Figure 6A, top panel), little is known regarding the extent of similarity in the range of sites
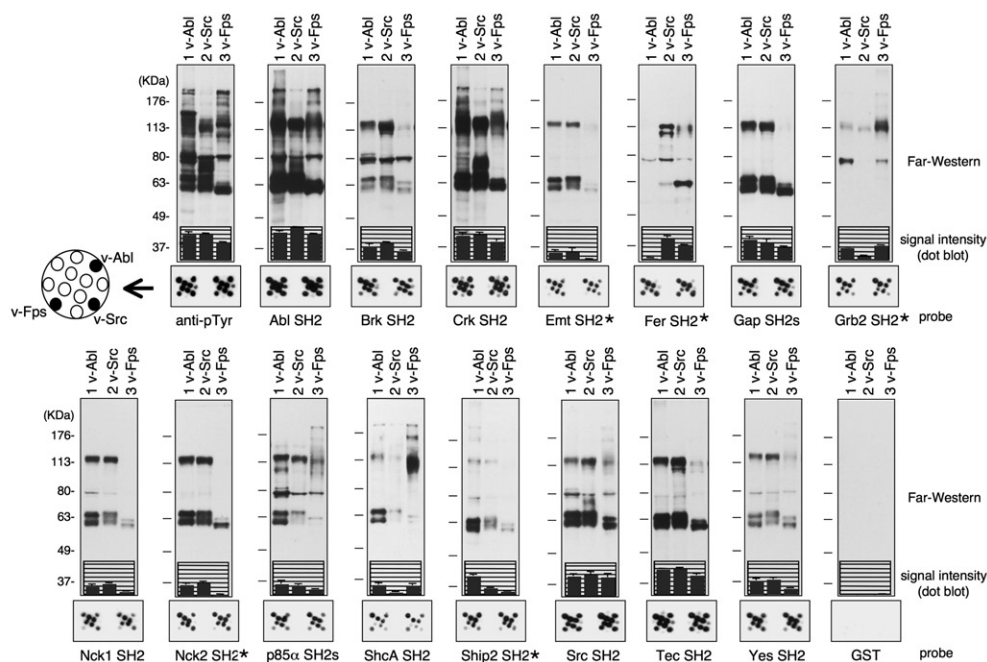
they phosphorylate in vivo (Kamps and Sefton, 1988). Whole-cell protein lysates from parental NIH 3T3; NIH 3T3 stably expressing v-Abl, v-Src, or v-Fps; NIH 3T3 overexpressing the CrkI and CrkII SH2/SH3 adaptors; and hepatocellular carcinoma HepG2 cells without or with pervanadate treatment (HepG2-POV) were immobilized, and the SH2 Rosette assay was performed (Figure 6A). We found that cells transformed by v-Src, v-Abl, and v-Fps were easily distinguishable on the basis of their binding preferences for individual SH2 domains. For example, as shown in the bar graph in Figure 6A (bottom panel, asterisks), the Fer SH2 bound relatively weakly to the v-Abl lysates, compared with v-Src and v-Fps lysates; similarly, the Grb2 SH2 bound relatively weakly to v-Src lysates, and the Emt and Nck SH2s bound relatively

Figure 5. Profiling SH2 Binding to Recombinant Phosphoproteins

(A) Schematic representation of PAK2 proteins used in the experiment. Amino acid sequences surrounding Tyr130, Tyr139, and Tyr194 are shown at the bottom.

(B) SH2 Rosette screening results for PAK2N. Purified PAK2N WT protein and positive control cell lysate (v-Abl-expressing 3T3 cells) were immobilized, and SH2 Rosette assay was performed. Quantified values (arbitrary units) for each probe are shown. Strong binding of Sap SH2 domain to PAK2N WT is indicated by arrow.

(C) Binding of Sap SH2 to PAK2N depends on pTyr194. Equal amounts of WT and mutant PAK2N proteins were separated by SDS-PAGE, transferred to membranes, and probed with indicated GST fusion probes or anti-phosphotyrosine antibody (pTyr). (−) indicates unphosphorylated (BL21-derived) protein; (+) indicates phosphorylated (TKB1-derived) protein.

weakly to v-Fps lysates. Thus global SH2 profiling revealed broad differences in the classes of tyrosine-phosphorylated sites present in cells transformed by different tyrosine kinase oncogenes.

We used 1D far-western blotting to confirm and extend the Rosette assay results. As expected, relative binding values obtained from dot blotting accurately predicted results seen in analytical far-western blotting (Figure 6B). Thus the SH2 Rosette assay provides a rapid, high-throughput method to identify differences among complex lysates that can then be subjected to more detailed

experimental analysis. For example, one could explore whether the relatively low level of Grb2 SH2 binding sites in Src-transformed cells versus Abl- and Fps-transformed cells reflects differences in the levels or mechanisms of activation of the Ras pathway in the different cell lines.

SH2 Rosette Assay for Profiling Adhesion-Dependent Cell Responses

To test whether this approach could be used to profile cells under different physiological conditions, we compared the SH2 binding profiles of adherent versus

suspended cells. For most adhesion-dependent cells, e.g., fibroblasts and epithelial cells, attachment to a solid substrate is essential for cell proliferation. When cultured in suspension, such cells undergo growth arrest often leading to apoptosis (termed anoikis); in contrast, tumor cells can survive and continue to proliferate. Several non-receptor PTKs including Src family tyrosine kinases (SFKs) are activated upon cell adhesion and mediate downstream signaling, including proliferation and survival signals (Giancotti and Tarone, 2003). To discern SFK-dependent phosphorylation events stimulated by cell adhesion, we utilized SYF cells, which are knockout-derived mouse embryonic fibroblasts lacking the Src, Yes, and Fyn SFKs, along with SYF cells rescued by re-expression of c-Src (SYF-Src).

Cells were cultured in suspension or adhesion conditions for 45 min or 24 hr, and the levels of tyrosine-phosphorylated binding sites for each SH2 domain were determined by Rosette assay (Figure 7A and Figure S5). The overall tyrosine phosphorylation level of SYF cells (assessed by pTyr antibody) was much lower compared to SYF-Src cells under all conditions, indicating the predominant role of SFKs in fibroblast cells. Adherent SYF-Src cells had elevated phosphorylation levels compared to cells in suspension, confirming that SFK-dependent phosphorylation occurs upon cell adhesion. Almost all SH2 domains showed enhanced binding in adherent cells, reflecting the global increase in phosphotyrosine. Particularly dramatic adhesion-dependent binding increases of more than 2-fold were seen for roughly one-fourth (11/45) of the SH2s analyzed (Figure 7A, asterisks).

We reasoned that SH2 binding sites that were highly dependent on adhesion were likely to play an important role in adhesion-mediated signaling, and we further investigated these by far-western analysis (Figure 7B). Different SH2 domains exhibited diverse patterns of binding to cellular phosphoproteins. For example, the Src SH2 bound to a wide range of phosphorylated proteins, closely mirroring the overall anti-pTyr pattern. On the other hand, the Cten and Csk SH2 domains each bound in an adhesion-dependent manner to a single predominant band of ~130 kDa and ~70–80 kDa, respectively.

We next attempted to identify the adhesion-dependent SH2 binding proteins using antibodies against proteins previously implicated in adhesion. Crk-associated substrate (p130Cas) and focal adhesion kinase (FAK) (both ~130 kDa) and paxillin (~70 kDa) were immunoprecipitated from lysates of adherent or suspended cells, and their overall tyrosine phosphorylation levels were deter-

mined with anti-phosphotyrosine antibody (Figure 7C). In each case, phosphorylation was enhanced by adhesion. The SH2 binding specificity for p130Cas, FAK, and paxillin was then determined by probing identical membranes with individual SH2 domains (Figure 7D and data not shown). These results are summarized in Figure 7E. Interestingly, the binding patterns of SH2s may be classified into five groups according to their ability to bind to these three proteins. Some SH2 domains, such as Fyn, Src, and Arg, bind strongly to all three proteins upon adhesion, while other SH2 domains are much more specific; for example Nck and Cten bind only p130Cas, and Csk binds only paxillin. Taken together, the SH2 Rosette assay and subsequent analyses provide insight into the network of interactions between SH2 domain-containing proteins and focal adhesion proteins that are likely to play a critical role in adhesion.

## DISCUSSION

SH2 Profiling and Phosphoproteomics

Given the importance of signaling mediated by tyrosine phosphorylation, there is great interest in strategies to define or profile the global state of tyrosine phosphorylation in the cell. Many tyrosine phosphorylation sites exert their biological activity through binding to other proteins containing SH2 domains, so by characterizing the SH2 domain binding activity of a cell we can very efficiently capture information relevant to the activation state of signaling pathways in that cell. In this report, we describe a flexible, high-throughput, quantitative SH2 profiling strategy. This approach requires minimal amounts of sample (including unpurified total cell lysate) and can serve as a first-step discovery tool to highlight tyrosine phosphorylation sites of potential interest that can then be further investigated by hypothesis-driven experimentation.

It is important to note that quantification of binding sites for the entire complement of SH2 domains, or SH2 profiling, provides two quite distinct kinds of information: (1) information that can be used to assign samples into classes that may be correlated with biological activities; and (2) information regarding the SH2 protein-phosphoprotein interactions that are likely to occur in vivo. Regarding the first type of information, we and others have proposed, for example, that SH2 binding profiles may be useful as a molecular diagnostic tool for classifying tumor cells and thereby predicting clinical outcomes (Dierck et al., 2006; Nollau and Mayer, 2001; Yaoi et al., 2006). For this purpose, the quantitative levels of binding sites for

Figure 6. SH2 Profiling of Oncogene-Transformed Cell Lines

(A) Analysis of PTK-expressing NIH 3T3 cellular lysates by SH2 Rosette assay. Left panels, photomicrographs showing morphology of NIH 3T3 cells expressing v-Abl, v-Src, and v-Fps PTKs. Middle panel, 400 ng (spot numbers 1–12) or 40 ng (spot numbers 13–24) of lysates from the following cells were probed with HRP-labeled GST-SH2 domains noted on right: NIH 3T3; NIH 3T3 stably expressing v-Abl, v-Src, v-Fps, CrkI, or CrkII; HepG2; pervanadate (POV)-treated HepG2 (HepG2-POV); and positive control (v-Abl-3T3-POV); or lysates were probed with antibodies for phosphotyrosine (anti-pTyr) or actin (anti-actin). Lower panel, average signal of duplicate spots was quantified (arbitrary units). Asterisks indicate probes that strongly discriminate among different PTK-transformed cell lines.

(B) Confirmation of SH2 Rosette results by far-western blotting. 1D gel-based far-western blot, and dot blotting-based result with quantitation, are shown for each SH2 probe along with GST control and anti-pTyr. Error bars indicate the SEM.
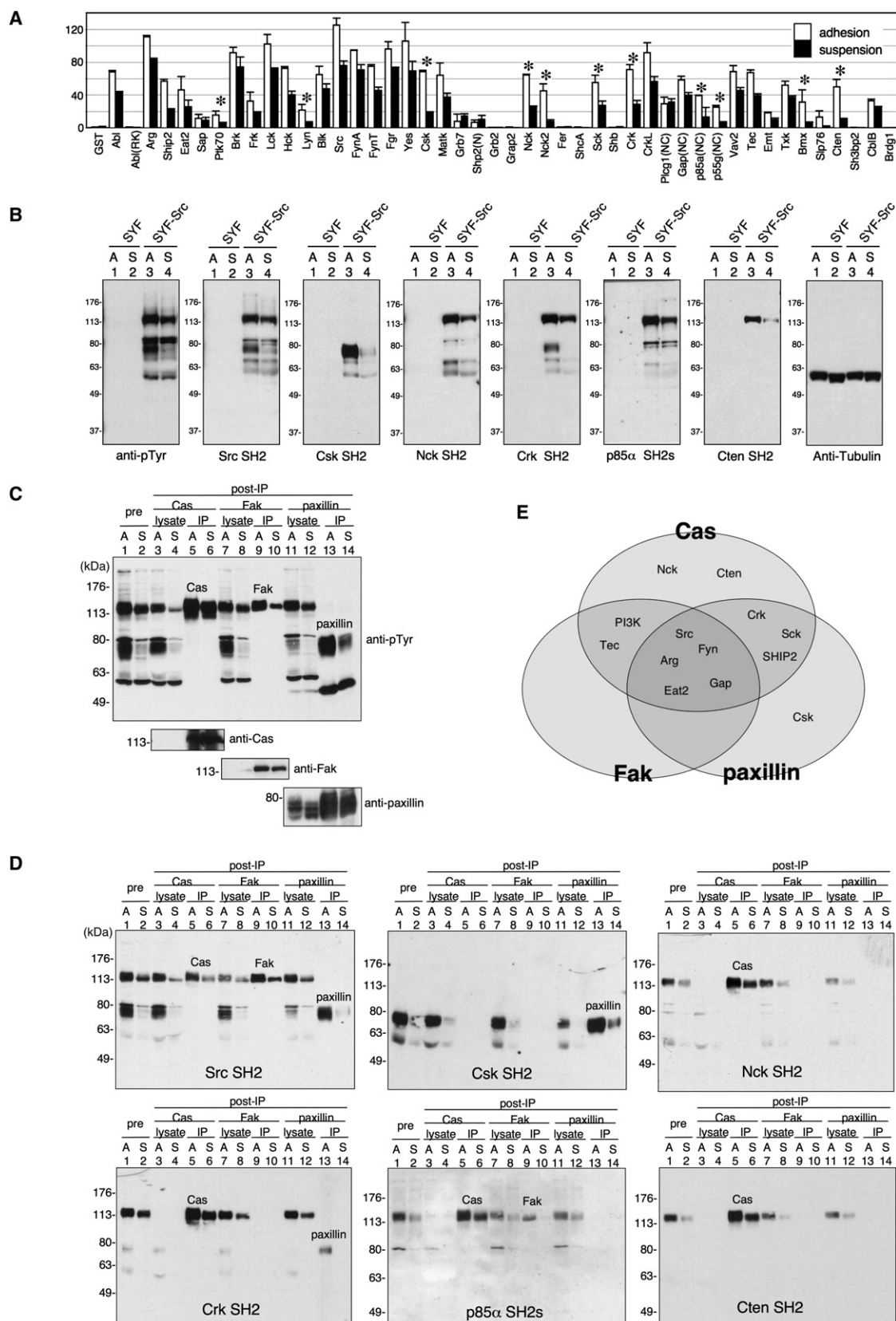
different SH2 domains serve only as a means of discriminating one class of samples from another (class discovery and class assignment). A particular quantitative pattern of SH2 binding may correlate, for example, with response to a particular therapy or risk of recurrence in certain classes of cancers. Although SH2 profile-based classification is likely to reflect actual biological differences in signaling state, SH2 binding data need not correspond to the actual physical interactions that occur in vivo. For example, quantifying binding sites for the SH2 domain of a protein that is not expressed in a tumor cell under analysis might still provide useful information for classifying that tumor.

Global SH2 binding patterns can also illuminate actual in vivo binding interactions and signaling pathway activity, thereby providing valuable information on signaling at the systems level. The signaling output of a tyrosine kinase pathway depends on a number of factors, including the specific protein sites phosphorylated, the absolute number and local concentration of those sites, their subcellular distribution, and the identity and local concentration of potential SH2-containing binding partners for those sites. Comprehensive SH2 profiling provides information about the overall concentration of binding sites for different SH2 domains, making it possible to predict what interactions are likely to occur under a given set of conditions. More importantly, changes in SH2 binding patterns provide information about which interactions are likely to change most dramatically upon signaling. For example, we have examined binding of SH2 domains to proteins from SYF-Src cells in adherent versus suspended conditions (Figure 7A). We find that for roughly 25% of the SH2 domains tested, adherent cell lysates had much higher levels of binding sites compared to suspended cell lysates. By focusing on those domains for which binding activity changed most dramatically, we rapidly identified a number of potential protein interactions that are dependent on, and therefore likely play a functional role in, the adhesion response. For example, we show that the Csk SH2 domain binds in a highly specific fashion to paxillin only in adherent cells. Although this interaction had been described previously (Sabe et al., 1994), only through comprehensive SH2 profiling analysis was the remarkable specificity and adhesion dependence of this interaction, compared to all other potential SH2-pTyr interactions, fully apparent.

Binding Properties of SH2 Domains

Our results also provide important insights into the general properties of SH2 domains. SH2 and PTB domains are the major known binding modules for tyrosine-phosphorylated proteins. PTB domains are entirely distinct from SH2 domains on the basis of primary sequence and structure, and only 25% of known PTB domains are thought to bind in a phosphorylation-dependent manner (Uhlik et al., 2005). For those SH2 domains with sufficient solubility and binding activity to assay, we did not encounter any example in which binding to total cellular proteins was not enhanced by tyrosine phosphorylation. These results indicate that the SH2 domain, in contrast to the PTB domain, represents a bona fide, dedicated phosphotyrosine recognition module. Of course, this analysis does not exclude the possibility that some SH2 domains may bind tightly to specific unphosphorylated proteins, as reported in several cases (Li et al., 1999; Mahajan and Earp, 2003; Pero et al., 2002).

We have determined binding profiles of 74 human SH2 domain probes using far-western analysis of pervanadate-treated cell lysates and quantified the relatedness of these binding patterns by hierarchical clustering. This analysis provides a comprehensive comparison of the binding patterns of SH2 domains for physiological mixtures of tyrosine-phosphorylated cell proteins. One advantage to the far-western approach used here is that it accurately reflects both the strength of binding to, and the relative abundance of, physiologically relevant binding partners, i.e., those found in actual cell lysates. This is quite distinct from (and complementary to) strategies using oriented random peptide libraries or peptide arrays to identify the preferred peptide binding sites for SH2 domains (Hwang et al., 2002; Songyang et al., 1993). Such experiments reveal the optimal binding sequence but do not provide information about actual protein binding sites present in cells. SH2 pull-down/MS approaches can identify specific SH2 binding sites in a sample (Blagoev et al., 2003) but provide little information on which sites are most abundant in that sample. By combining data obtained through each of these approaches, along with expression data for SH2-containing proteins and potential partners, it will be possible to construct an accurate and comprehensive map of all SH2-mediated interactions in the cell under different physiological conditions.

Figure 7. SH2 Profiling of SYF Cells in Adherent and Suspended States

SYF cells or c-Src reconstituted SYF cells (SYF-Src) were cultured in adhesion or suspension conditions for 45 min (see Figure S5 for details).

(A) Quantitative SH2 Rosette binding data (arbitrary units) for adherent (white bar) or suspended (black bar) SYF-Src cells. Asterisks indicate more than 2-fold higher binding in adherent versus suspended cells. Error bars indicate the SEM.

(B) Far-western blotting with Src, Csk, Nck, Crk, p85α, and Cten SH2 domain probes, and immunoblotting with anti-pTyr and anti-tubulin. A and S denote adherent and suspended cells, respectively.

(C) Identification of adhesion-dependent SFK substrates. SYF-Src lysates were subjected to immunoprecipitation (IP) with p130Cas, FAK, and paxillin antibodies. Lysates before or after IP (indicated as pre- and post-IP, respectively), and precipitates (IP), were immunoblotted with anti-phosphotyrosine antibody (upper panel) or antibodies for Cas, FAK, and paxillin (lower panels).

(D) Identical filters to those in (C) were probed with SH2 domains of Src, Csk, Nck, Crk, p85α, and Cten.

(E) SH2 domain probes were classified into five groups according to their ability to bind p130Cas, FAK, and paxillin in adherent SYF-Src cells ([D] and data not shown).

The comprehensive far-western binding data we have obtained enables us to compare the overall binding patterns for different families of SH2 domains. In general, we find that SH2 domains of nonreceptor tyrosine kinases recognize a broad spectrum of binding sites in the cell, while other SH2 domains, particularly those from adaptor proteins such as the Grb2 family, often recognize a more limited set of phosphoproteins. This is consistent with a general role for SH2 domains in tyrosine kinases in binding to a wide range of kinase substrates and thereby facilitating their sustained, processive phosphorylation (Duyster et al., 1995; Mayer et al., 1995; Songyang et al., 1995). SH2 domains of adaptors, on the other hand, are likely to have evolved to mediate more specific protein complexes (Pawson, 2004).

We have also compared the similarity of SH2 domains by sequence alignment with their similarity based on binding profile (Figure 2B). Except for closely related protein families, the correlation between evolutionary distance and binding pattern is weak. This is not surprising, because NMR and X-ray crystal structures have shown that only a few residues of the SH2 domain directly contact the ligand and determine specificity; thus for more distantly related domains, most of the sequence differences are irrelevant to binding. We note, however, that it should be possible to use our binding data to identify those residues that correlate most strongly with binding specificity, which are likely to directly participate in peptide binding. We performed a pilot experiment in which we aligned SH2 domains based only on residues predicted from structural studies to contact ligand. In this analysis, NAP4 and other SOCS family members clustered relatively close to the Src family (data not shown), consistent with our binding studies showing that the NAP4 and Src family SH2s have very similar binding profiles (Figure 2). Songyang et al. previously proposed to classify SH2 domains into four groups based on the "specificity determining" βD5 residue (Songyang et al., 1993). We found no obvious correlation, however, between these four groups and our SH2 binding-based clusters (data not shown).

Another important aspect of binding specificity is the extent to which there is a one-to-one correspondence between a particular phosphorylated site and a particular SH2 domain. This is an important question for understanding the "wiring" of signaling networks. Currently, there is often an implicit assumption that a particular phosphorylation site binds only to a single SH2 domain, despite the relatively limited binding specificity of SH2 domains and the relatively large number of SH2 proteins expressed in the cell. Our data indicate that while specificity varies with the site, in some cases many SH2 domains can compete on roughly equal terms for binding to the same sites. This was most specifically addressed by competitive binding assays in the case of specific PECAM phosphorylation sites (Figure S3). Fifteen SH2 domains bound with reasonably high affinity to the Tyr663 and/or Tyr686 sites, and in virtually all cases binding of any single

SH2 domain was strongly inhibited by a cocktail of unlabeled SH2 domains, suggesting multiple domains bind with similar affinities. Only in one case, the binding of the Shp2 SH2(N) to the Tyr663 site, was binding largely resistant to inhibition by competition. Thus we can conclude that binding of Shp2 SH2(N) to this site would be strongly favored in vivo. In the case of Tyr686, which SH2 protein binds in vivo will be strongly affected by the local concentrations and availability of the competing domains.

Features of the SH2 Rosette Assay

To generate comprehensive, quantitative, high-throughput SH2 domain binding data using minimal amounts of sample, we adopted a reverse-phase array (dot-blotting) format. A variety of methodologies have been developed recently to profile the binding of SH2 domains to tyrosine-phosphorylated samples, and a detailed discussion of the comparative advantages and disadvantages of the different SH2 profiling platforms is presented in the Supplemental Data. An important practical difference between the Rosette method and forward-phase methods (where SH2 domains are arrayed on a surface or coupled to coded beads, and the analyte is in solution) is in their binding kinetics. Forward-phase assays work best for purified analytes such as synthetic phosphopeptides, where the high concentrations needed to drive efficient binding to immobilized SH2 domains can be achieved. This approach can also provide the dissociation constants for binding of the analyte to each SH2 domain in the array (Jones et al., 2006). Reverse-phase assays, however, are better for analyzing complex samples containing relatively low amounts of phosphorylated targets, such as unfractionated cell lysates. In this case, high concentrations of the SH2 domains in solution can be used to drive binding to the immobilized sample.

Two of the strengths of the SH2 Rosette approach are its wide applicability and its sensitivity. We have shown that many types of samples including whole-cell lysates can be profiled, and the number of samples and of SH2 domains per assay is flexible and easily adjusted. The assay uses the same detection methodology and reagents as 1D far-western-based SH2 profiling (Nollau and Mayer, 2001), which we previously reported is at least as sensitive as anti-phosphotyrosine immunoblotting, depending on the specific SH2 domain and sample (Nollau and Mayer, 2001). We show here that sensitivity of the SH2 Rosette assay is more than sufficient to profile lysates of cells with relatively normal levels of tyrosine phosphorylation, such as SYF-Src cells (Figure 7). The fact that SH2 domain probes are fused to GST, which dimerizes in solution, and are detected by binding to highly oligomeric glutathione-HRP conjugate, likely increases assay sensitivity through avidity effects as earlier noted for far-western blotting (Nollau and Mayer, 2001).

We were initially concerned that the quantitative dot-blotting format might result in loss of specificity compared with 1D far-western blotting, but our results indicate that this is not a significant obstacle. We found the overall

signal-to-noise ratio to be similar for dot-blotting and 1D gel formats, and ratiometric signal intensities are comparable (Figure 6B). And although some differences in binding patterns will inevitably be obscured in dot-blotting compared to 1D gel format (especially when the overall binding levels for two samples are similar), this disadvantage is more than offset by much higher throughput. Furthermore, any potentially interesting results can easily be subjected to far-western analysis in a two-step screening strategy.

Although this format is designed for highly sensitive quantification of SH2 binding sites, as opposed to the direct identification of SH2 ligands, if identification of specific binding partners is needed this can be accomplished through additional analysis. We have shown that binding partners can be identified in some cases by antibody-based screening of candidate proteins (Figure 7D). Alternatively, unbiased identification of proteins is possible using affinity purification and MS.

Taken together, the strategy of comprehensive screening by SH2 Rosette assay in combination with 1D analysis is a flexible, sensitive, and specific approach to profile the global tyrosine phosphorylation state. Because it is technologically straightforward and can be performed on small amounts of unpurified sample, it offers an unprecedented opportunity to analyze the tyrosine phosphoproteome in a wide variety of research and clinical applications.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Proteins

cDNA clones for the human complement of SH2 domains (Liu et al., 2006) were purchased from ATCC except for those cloned by RT-PCR from a mouse cDNA library (Lnk, Sck, Syk, and Txk) or as noted in the Acknowledgments. SH2 domains were cloned into pGEX fusion vectors (Amersham Pharmacia) and verified by sequencing. GST-SH2 fusion proteins were purified as previously described and stored in small aliquots at −80°C (Mayer et al., 1991; Nollau and Mayer, 2001). Detailed information for each construct is available in Table S2. For PAK2N-H6 proteins, amino acids 1–212 of the PAK2 tyrosine mutants (Renkema et al., 2002) were subcloned into the pET23d vector (Clontech). Expression was induced in BL21(DE3)pLysS cells or TKB1 cells (both Stratagene) and the proteins were purified using Ni-NTA agarose beads (QIAGEN) according to the manufacturer's instructions.

Sample Preparation

To generate a broad-spectrum tyrosine-phosphorylated cell lysate for comparing SH2 binding patterns (Figure 1B), equal amounts of lysate from pervanadate-treated NIH 3T3, HepG2, A431, and MR20 cells were combined. As a control, lysates of each cell line were prepared in the absence of vanadate, combined, and treated with tyrosine phosphatase PTP-1B for 1 hr at room temperature (RT). Proteins were precipitated with 3 vol cold ethanol overnight, redissolved in SDS sample buffer (180 mM Tris-HCl [pH 6.8], 30% glycerol, 6% sodium dodecyl-sulfate [SDS], 15% β-mercaptoethanol, and 0.03% bromophenol blue), and boiled for 3 min prior to gel electrophoresis or dot blotting. More details of cell culture and lysis conditions are available in the online Supplemental Data.

Characterization of SH2 Binding Profiles

GST-SH2 domains were labeled with horseradish peroxidase-conjugated glutathione (GSH-HRP) and far-western blotting was performed as previously described (Nollau and Mayer, 2001). Conditions were in-dividually optimized for each probe (final SH2 probe concentrations ranged from 10–140 nM; see details provided in Table S2). To compare SH2 binding patterns (Figure 1B), identical blots of pervanadate-treated mixed lysate were prepared using precast polyacrylamide gels (NuPAGE 4%–12%, Invitrogen) and a multigel apparatus (Xcell6 unit, Invitrogen). Replicate experiments for each domain were performed on different days using different gels and membranes. Signals were detected with Lightning Chemiluminescence Reagents Plus (PerkinElmer) and exposed to X-ray films. Developed films were scanned, and signal intensities of whole lanes were quantitated by densitometry (ImageJ 1.33) after background subtraction. Far-western gel lanes with roughly equivalent signal levels were selected, and when necessary image sizes were adjusted to align precisely, using images of the same filters reprobed with anti-pTyr as a reference. Aligned gel lanes were sliced into 48 bins, and signal in each bin was quantified. Each densitometry-quantified protein lane was projected as a vector into a high-dimensional space, and the Euclidean distance between these vectors was calculated as the pairwise binding pattern distance. Hierarchical clustering analysis was performed using Cluster v3.0 (Eisen et al., 1998) with average linkage clustering and absolute correlation values. The result was visualized using Java TreeView v1.1.0 (Saldanha, 2004).

Sequence Alignment Quantification

The SH2 domain alignment was generated as described (Liu et al., 2006). Briefly, the 120 SH2 domain borders determined by SMART/Pfam were extended in the N-terminal and C-terminal ends by 10–15 amino acids. The sequences were analyzed by ClustalW using Bioedit or Mega with settings containing a full multiple sequence alignment with parameters set at pairwise gap open, 0; extend, 0; multiple alignment gap open, 1; extend, 0. The ClustalW alignment was run in addition with settings including neighbor joining tree with bootstrap settings at 1000. The protein distance matrix (pairwise) was compiled through the distance matrix software from the ClustalW alignment integrated in the Bioedit/Mega software package.

Dot Blotting

Peptides were immobilized to gelatin-coated nitrocellulose membranes by 4% paraformaldehyde treatment as described (Too et al., 1994). Lysates were spotted onto nitrocellulose membranes (BA83, Schleicher & Schuell BioScience) at 0.1 μl per spot using gel loading tips guided by a transparent template on a light box. Dried membranes were soaked in buffer A (10 mM CAPS [pH 11.0] and 20% methanol) for 30 min, rinsed once with TBST (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], and 0.05% Tween-20), and blocked for 1 hr at RT in TBST containing 10% nonfat milk, 1 mM Na_3VO_4, and 1 mM EDTA. Each GST-SH2 probe was labeled with GSH-HRP and applied to a separate well of a 96-well chamber plate (MBA96, Neuro Probe) assembled with a blocked membrane. After 1 hr of incubation at RT, chambers were separately washed three times with TBST, and then the entire filter was washed for 15 min.

Supplemental Data

Supplemental Data include supplemental text, Supplemental Experimental Procedures, Supplemental References, five figures, and three tables and can be found with this article online at http://www.molecule.org/cgi/content/full/26/6/899/DC1/.

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FEBS Letters

POSH2 is a RING finger E3 ligase with Rac1 binding activity through a partial CRIB domain

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ABSTRACT

The Plenty of SH3 domains protein (POSH) is an E3 ligase and a scaffold in the JNK mediated apoptosis, linking Rac1 to downstream components.

We here describe POSH2 which was identified from a p21-activated kinase 2 (PAK2) interactor screen. POSH2 is highly homologous with other members of the POSH family; it contains four Src homology 3 (SH3) domains and a RING finger domain which confers E3 ligase activity to the protein. In addition POSH2 contains an N-terminal extension which is conserved among its mammalian counterparts. POSH2 interacts with GTP-loaded Rac1. We have mapped this interaction to a previously unrecognized partial Cdc42/Rac1-interactive binding domain.

Structured summary:
MINT-7987761: *POSH1* (uniprotkb:Q9HAM2) *physically interacts* (MI:0915) with *Ubiquitin* (uniprotkb:P62988) by *anti tag coimmunoprecipation* (MI:0007)
MINT-7987932: *PAK2* (uniprotkb:Q13177) *binds* (MI:0407) to *CDC42* (uniprotkb:Q07912) by *solid phase assay* (MI:0892)
MINT-7987908: *POSH1* (uniprotkb:Q9HAM2) *binds* (MI:0407) to *Rac1* (uniprotkb:P63000) by *solid phase assay* (MI:0892)
MINT-7987880: *POSH2* (uniprotkb:Q8TEJ3) *binds* (MI:0407) to *Rac1* (uniprotkb:P63000) by *solid phase assay* (MI:0892)
MINT-7987734: *POSH2* (uniprotkb:Q8TEJ3) *physically interacts* (MI:0915) with *Ubiquitin* (uniprotkb:P62988) by *anti tag coimmunoprecipation* (MI:0007)
MINT-7987779, MINT-7987804, MINT-7987824, MINT-7987838, MINT-7987853: *Rac1* (uniprotkb:P63000) *physically interacts* (MI:0915) with *POSH2* (uniprotkb:Q8TEJ3) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-7987920: *PAK2* (uniprotkb:Q13177) *binds* (MI:0407) to *Rac1* (uniprotkb:P63000) by *solid phase assay* (MI:0892)

1. Introduction

Protein–protein interactions domains play important roles in many cellular processes. To perform their proper functions, proteins have to be directed to specific cellular structures and locations and have to be connected to different interaction partners.

One common interaction domain is the Src homology 3 (SH3) domain [1,2]. The human genome contains about 217 SH3-domain containing proteins with in total almost 300 different SH3 domains [3].

The ligand of SH3 domains is the so called left-handed polyproline (PPII) helix which is a short peptide, usually containing several proline residues and referred to as the PxxP-motif (reviewed in [4]).

The p21-activated kinases (PAKs) are serine–threonine kinases involved in many cellular processes (reviewed in [5]). Major roles have been studied in cytoskeletal rearrangements [6], immunology and viral pathology [7], and the p21-activated kinases are studied as targets for cancer therapy [8,9]. The activity of these

kinases is regulated through an auto-inhibitory mechanism [10] by which the catalytic cleft of the kinase domain is occupied by part of the N-terminal regulatory domain in a *trans* manner. The N-terminal domain has two functions. It serves as an inhibitor of the kinase domain, but it also contains several sites for interactions with other proteins, such as the Cdc42/Rac-interactive binding (CRIB) motif for the interaction with the small PAK-activating GTPases Cdc42 and Rac, but also several PxxP-motifs are found [5].

In a screen of all SH3 domains present in the human proteome for p21-activated kinase interacting SH3-domain containing proteins we identified the third SH3 domain of a protein we called POSH2 [3]. POSH2, like Plenty of SH3 domains (POSH, [11]) contains four SH3 domains, as well as a RING (Really Interesting New Gene) finger domain. More recently, Wilhelm et al. identified and cloned a similar protein from rat by BLAST homology searches using the POSH sequence and named this protein POSH2 [12]. However this protein is not the ortholog of the human POSH2 we identified and should therefore not have been named the same. For clarity we will refer to the Wilhelm et al. protein as POSH3, the original POSH identified by Tapon et al. [11] we will hereafter refer to as POSH1.

POSH1 is the most studied member of this protein family so far. POSH1 was originally identified as a novel Rac1 binding protein from a yeast two-hybrid screen, containing a novel GTPase interaction domain [11]. The N-terminal RING finger domain confers E3 ubiquitin ligase activity to the protein [13] and the four SH3-domain are critical for interactions with other proteins. In mammalian cells POSH1 was found to function as a scaffold in a multiprotein complex that links activated Rac1 and downstream elements of the JNK apoptotic cascade [13].

In this paper we explore some of the characteristics of POSH2. POSH2 was found to have a conserved N-terminal extension not present in POSH1 and POSH3. We show that POSH2 has GTP-loaded Rac1 binding activity and we have mapped this activity to a partial CRIB domain.

2. Materials and methods

2.1. Plasmids

The POSH2 fragments, POSH2 (amino acids (AA) 1–70) and POSH2 (AA46–70) were cloned into pEGFP-N1 vector (Clontech) 5′ of green fluorescent protein (GFP) with their natural Kozak using upstream primers 18 nucleotides 5′ of the ATG.

The minimal Rac-binding site of POSH1 (AA292–362) [11] and the same region from POSH2 (AA369–439) were cloned in frame with Glutation-*S*-transferase (GST) into pEBG vector. Mutations in pEBG-POSH2 (AA369–439) were generated using overlap PCR and mutagenic oligo's, yielding the mutants I403N/S404P/P406A (ISP), I403N, S404P, L385A, and P414 (amino acid numbers according to the full length sequence).

The N-terminus of PAK2 (PAK2N) (amino acids 1–251) was cloned into bacterial expression pGEX 4T-1 vector (GE Healthcare).

Cdc42 and Rac1 were cloned into pGEX-PP containing an N-terminal biotin purification tag (BPT) [14]. The constitutively GTP-loaded Rac1V12 was cloned into pEBB with an N-terminal Myc-epitope tag.

pMT-123-Ubi-HA (Ubi-HA) was kindly provided by Daniela Ungureanu, Tampere. Full length POSH1 (NCBI Genbank reference sequence NM_020870) and POSH2 were cloned into pEBB vector containing N-terminal Flag tag. The ring domain mutations, CH-AA (POSH1 C28A/H30A, POSH2 C73A/H75A) and the CRIB domain mutant (S404P), were made by overlap PCR using mutagenic primers.

The 3xAP-1-luc reporter construct was kindly provided by Kalle Saksela, University of Helsinki.

2.2. Antibodies

The following antibodies were used: mouse anti-c-myc (Roche), mouse anti-Flag M2 (Sigma), mouse anti-HA (Nordic BioSite AB), mouse anti-GFP (Zymed), goat anti-GST (Amersham Biosciences). Secondary antibodies used: biotinylated polyclonal goat anti-mouse and biotinylated polyclonal rabbit anti-goat (both from DakoCytomation).

2.3. Cell culture and mammalian cell transfections

HEK293T cells (ATCC) and osteosarcoma U2OS cells (kind gift from Pekka Lappalainen, Helsinki) were cultured using standard procedures in Dulbeccós modified Eaglés medium (DMEM; Lonza) supplemented with 10% fetal calf serum (FCS; Sigma), 2 mM L-Alanyl-L-Glutamine (Biochrom), 1% penicillin–streptomycin (Sigma–Aldrich) at 37 °C with 5% CO_2. HEK293T cells were transfected using TurboFect reagent (Fermentas) or by Ca-phosphate precipitation [15] in the case of recombinant protein production for the overlay assays. For immunofluorescent assays U2OS cells were grown on cover slips and transfections were performed using TransIT-LT1 transfection reagent (Mirus) according to the manufacturer's instructions.

Cell lysates were made as described before [16]. Transfection efficiency was monitored by determining beta-galactosidase activity from a co-transfected pEBB-LacZ plasmid using an *o*-nitrophenyl-β-D-galactopyranoside assay [17].

2.4. Immunoprecipitation and immunoblotting

For the ubiquitination assays and Rac co-immunoprecipitation experiments Protein A-Sepharose CL-4B beads (GE Healthcare) were incubated with antibodies (anti-Flag or anti-Myc, respectively) and cell extracts (350 or 700 μg protein, respectively) for 3 h at 4 °C. After washing three times with lysis buffer the beads were boiled in sample buffer followed by SDS–PAGE. Proteins were transferred to nitrocellulose membrane (Perkin–Elmer Life Sciences) and analyzed by western blotting. Detection of the biotinylated secondary antibodies was done with streptavidin–biotinylated horseradish peroxidase (SA-HRP) (GE Healthcare) and enhanced chemiluminescence (ECL, Millipore). The quantitative data was produced using Bio-Rad Quantity One Software version 4.5.2.

2.5. Expression and purification of recombinant proteins

Recombinant GST and GST-fusion proteins were produced either in *Escherichia coli* bacteria (GST, GST-PAK2N, GST-BPT-Cdc42, and GST-BPT-Rac1) or in HEK293T cells (GST-POSH1 (AA292–362) and GST-POSH2 (AA369–439)). Bacterial protein production was induced with1 mM isopropyl β-D-1-thiogalactopyranoside (4 h). Cells were lysed in PBS/1% Triton X-100 and complete protease inhibitor cocktail using sonication. Transfected HEK293T cells were lysed 48 h after transfection. Cleared lysates were incubated with Glutathione Sepharose 4B beads (GE Healthcare). After washing (1× PBS/1% Triton X-100, 2× PBS/0.1% Triton X-100, 1× PBS), proteins were eluted (100 mM Tris (pH 9.8)/20 mM glutathione) and dialyzed against PBS (4 °C over night) or concentrated in PBS by Amicon Ultra-4 centrifugal filter columns (Millipore). Protein concentration was assayed using the DC Protein Assay (Bio-Rad) and the integrity of the purified proteins was checked on SDS–PAGE gels using Coomassie blue.

2.6. GTPase overlay assay

Adaptated from Tapon et al. [11]. 20 μg of GST, GST-POSH1 (AA292–362), and GST-POSH2 (AA369–439) and 2.5 μg of GST-PAK2N were spotted on nitrocellulose strips. The strips were air-dried and blocked (4 °C over night) in 1 M glycine/5% milk powder/5% fetal calf serum. 4 μg purified GST-BPT-Cdc42 or GST-BPT-Rac1 were loaded with nucleotide in 50 μl 25 mM Tris (pH 7.4)/0.1 M NaCl/5 mM EDTA/1 mM dithiotreitol including 0.2 mM guanosine 5′-O-(3-thiophosphate) tetralithium salt or guanosine 5′-diphosphate sodium salt (Sigma–Aldrich) (10 min, 30 °C) and stopped by the addition of $MgCl_2$ up to 6 mM. The loaded GTPases were then labeled (on ice, 15 min) with 2 μl SA-HRP. The blocked strips were washed in buffer A (50 mM Tris (pH 7.5)/100 mM NaCl/5 mM $MgCl_2$/0.1 mM dithiothreitol and incubated (30 min, 4 °C) with the indicated nucleotide loaded GTPases. The strips were washed three times for 5 min with 3 ml of cold buffer A/0.1% Tween. Biotin-labelled GTPases bound to the membrane were visualized by ECL.

2.7. Luciferase reporter assay

Plasmids for full length POSH2, both wild type and S404P mutant, were transfected to 293HEK cells together with an AP-1 report construct (3xAP-1-luc, containing three binding sites for AP-1) and the lacZ transfection control plasmid in a 24 wells format. Two days after transfection, the cells were lysed in 150 μl Luciferase Cell Culture Lysis Reagent (Promega). Lysates were cleared by centrifugation (5 min 13,000×g) and parts of the lysates were used for beta-galactosidase assays and luciferase assays using Luciferase Assay Reagent (Promega). Luciferase measures were corrected over the transfection efficiency. Significance of the data was calculated using a Student t-test.

3. Results and discussion

3.1. The POSH family has three mammalian members

We have cloned the full length coding sequence for the human POSH2. The POSH2 cDNA encodes for a protein of 882 amino acids which has a vast degree of homology to POSH1 (46% identity) and POSH3 (33% identity). An alignment of the human and mouse or rat POSH1–3 can be found in the Supplementary material as well as cloning details. Extensive database searches using the CDART and BLAST searches from NCBI have not revealed more members of this protein family.

POSH2 contains the hallmark $Cys_3HisCys_4$ residues of the RING finger domain that is also found in POSH1 and POSH3. Between the second and the third SH3 domain there is a conserved region in all POSH members. Part of this region in POSH1 (amino acids 292–362) has previously been found to contain a minimal Rac-binding site [11]. The SH3 domains, comprising a major part of the POSH proteins, establish interactions with PxxP-motif containing proteins. POSH1 and POSH2 both contain 4 SH3 domains but POSH3 lacks the 4th SH3 domain [12], likely having an effect on the panel of interacting proteins.

POSH2 is highly conserved in mammals, but a POSH2 like sequence was also found from *Gallus gallus* (hypothetical protein XP_416930) although the N-terminus of this protein is either different or not correctly predicted from the genomic sequence. Microarray expression data of the human POSH2 is available from the Weizmann Institute of Science GeneNote service, and shows POSH2 expression in thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas, and prostate. The currently available expression patterns for the POSH pro-

teins are not directly comparable due to the different tissues studied [11,12], but in general these proteins can be considered as ubiquitously expressed. Detailed comparison of expression patters and interaction partners would be of interest since at this moment no data is available on possible functional redundancy of the different POSH proteins.

In a recent paper by Votteler et al. [18] the authors found that ALIX-mediated release of infectious HIV virions was enhanced by POSH1 although silencing of POSH1 by RNAi did not have the anticipated opposing effect. In the light of the presence of three POSH proteins in mammalian cells it is however possible that knockdown of all POSH family members would give the expected results.

It is relevant to note that according to our database searches, *Drosophila melanogaster* and other insects have only one POSH gene.
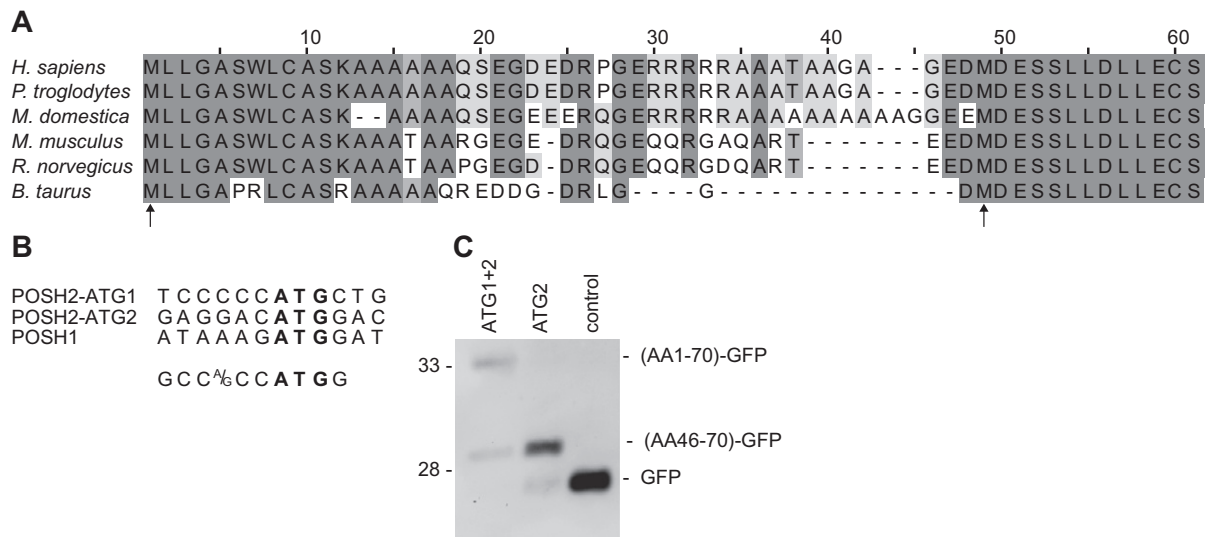
3.2. POSH2 from different species contains an N-terminal extension

Surprisingly POSH2, in contrast to POSH1 and POSH3, has no in frame stop codon 5′ to the conserved methionine that precedes the RING finger domain, however, there is an in frame start codon. The sequences surrounding either ATG codon are not perfect Kozak consensus sequences for translational start (Fig. 1B) so the start of the protein coding sequence is not immediately apparent. BLAST searches showed that the in frame upstream start codon is present in POSH2 sequences from different species and the translated amino acid sequence is conserved although the length of the extension varies from 46 amino acids in humans to 28 amino acids in *Bos Taurus* (Fig. 1A).

In order to determine which one is the preferred ATG for protein translation in the POSH2 protein we made expression plasmids encoding GFP preceded with and N-terminal fragment of POSH2 starting either from the first or from the second ATG. Both constructs contain the natural Kozak sequence as is found from the mRNA. As is shown in Fig. 1C, the construct starting from the second ATG (ATG2) produces a protein of the expected molecular weight, although a small amount of only GFP was detected in this lane probably due to proteolytic cleavage. The construct that starts from the first ATG, and therefore contains both ATGs (ATG1+2), produced the longer protein (AA1–70) GFP as well as small amounts of the (AA46–70) GFP. Quantification of the expression levels from six different experiments showed a ration of 63:37 of the usage of the first ATG over the second ATG. We therefore concluded that the first ATG is a bona fide start site for protein translation, but significant usage of the second site was also observed. The possible function for the N-terminal extension of POSH2 to act as an N-terminal targeting sequence was excluded (see Supplementary data).

3.3. POSH2 has ubiquitin ligase activity

The RING finger domain of POSH1 was found to transfer E3 ligase activity to the protein [13]. Like POSH1, POSH2 contains all the characteristic amino acids of the RING finger motif. We therefore decided to mutate two of the critical amino acids to study the possible E3 ligase activity of POSH2. The wild type and mutant constructs were transfected to 293 HEK cells in the absence or presence of co-transfected Ubi-HA plasmid. Transfection efficiencies were monitored by determining beta-galactosidase activity from a co-transfected pEBB-LacZ plasmid using a colorimetric assay. However, this is also a measure for the survival of the transfected cells. As shown in Fig. 2, we found that in the case of transfection of the wild type POSH1 or POSH2 together with the Ubi-HA plasmid resulted in a marked reduction of the expression of beta-galactosidase suggesting that under these conditions there is a

Fig. 1. POSH2 contains a conserved N-terminal extension. (Panel A) Alignment of the (predicted) amino acid sequences of the N-terminal extension of POSH2 proteins from different species. Shading of amino acids is as follows: dark grey; identical in 5 or more sequences, middle grey; identical in 4 sequences, light grey; identical in three sequences. Both methionine residues are indicated with arrows. (Panel B) Alignment of both POSH2 ATG start codons with the POSH1 start codon and the Kozak consensus sequence (bottom row). ATG codons are indicated in bold. (Panel C) HEK293T cells were transfected with either POSH2 (AA1–70)-GFP (ATG1+2), POSH2 (AA46–70)-GFP (ATG2) or plain GFP. Western blot analysis of cell lysates using an anti-GFP antibody is shown. Mobility of the different proteins and molecular weight markers (kDa) are indicated.
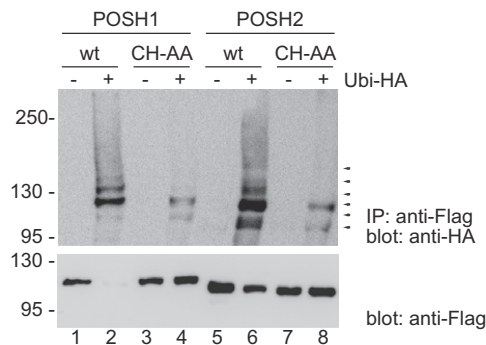
Fig. 2. POSH2 self ubiquitination depends on the RING finger domain. HEK293T cells were transfected with Flag-tagged wild type or RING finger mutant (CH-AA) POSH1 and POSH2 with co-transfected Ubi-HA when indicated. Part of the cell lysates was used for Western blot analysis of the expression of POSH using the anti-Flag antibody (lower panel) the remaining was immunoprecipitated using anti-Flag followed by anti-HA Western blotting to detect ubiquitinated POSH (upper panel). Arrow heads indicate the different ubiquitinated forms of POSH. Molecular weight markers are indicated (kDa).

quite significant loss of transfected cells, likely due to apoptosis. Concomitantly, the protein expression levels of both the wild type POSH1 and POSH2, as seen in the anti-Flag blot, were decreased. The small amounts of remaining POSH protein, however were found to be massively ubiquitinated (upper panel). Mutation of C73 and H75 for POSH2 and C28 and H30 for POSH1 into alanine residues (CH-AA mutants) resulted in marked reduction of the level of ubiquitination for both POSH1 and POSH2 indicating that these residues in POSH2 are indeed involved in self-ubiquitination of the protein. The transfected protein levels of the mutant POSHs in the Ubi-HA co-transfection was improved as well as the transfection/survival efficiency, especially in the case of POSH1 but also for POSH2.

In the absence of the Ubi-HA we did not see consistent differences in the protein amounts between the wild type and RING mutants of both POSHs. This is in disagreement with result published by Xu et al. [13] who found decreased protein levels of the wild type POSH1 in the absence of a construct that enhances ubiquitination. We have at the moment no explanation for these differences, but speculate that there are differences in the self-ubiquitination and subsequent stability of POSH in the different experiments, although the same cell line was used.

From this experiment we concluded that POSH2 indeed contains a functional RING finger domain that transfers E3 ligase activity to this protein.

3.4. The POSH2 RBD consists of a partial CRIB domain

POSH1 was originally identified as a Rac GTPase binding interacting protein from a yeast 2-hybrid screen using L61 Rac as bait [11]. POSH1 did not bind to Rho and Cdc42 and only bound Rac when it was loaded with GTP. Tapon et al. determined that the minimal Rac binding domain (RBD) of POSH1 was a region of 70 amino acids (AA292–362). The authors did not find sequence homologies with other GTPase binding proteins and therefore named it a unique GTPase binding site. We performed GTPase overlay experiments to determine whether this region in POSH2 also contains GTPase binding activity. As shown in Fig. 3A, GST-POSH2 (AA369–439) bound GTP-loaded Rac1 comparable to GST-POSH1 (AA292–362). The POSH2 domain bound in a GTP-dependent manner and was specific for Rac1 since no binding to GDP-loaded Rac1 and GTP-loaded Cdc42 was detected. Although our detection limit does not allow to conclude the absence of an interaction with the GDP-loaded Rac1 the difference with the GTP-loaded GTPase is obvious and in line with results obtained with the POSH1 fragment [11].

From our alignment of POSH1, 2, and 3 we noted that the region responsible for Rac binding is highly conserved. In fact, part of the region shows similarity with part of the CRIB (Cdc42- and Rac-interactive binding) domain with the consensus sequence I-S-X-P-(X)$_{2-4}$-F-X-H-X-X-H-V-G [19]. In Fig. 3B we aligned the CRIB domains of proteins known to interact with either Cdc42 or Rac [19] with the sequences of POSH. The Rac interacting region in POSH contained the N-terminal I-S-X-P sequence of the CRIB domain.
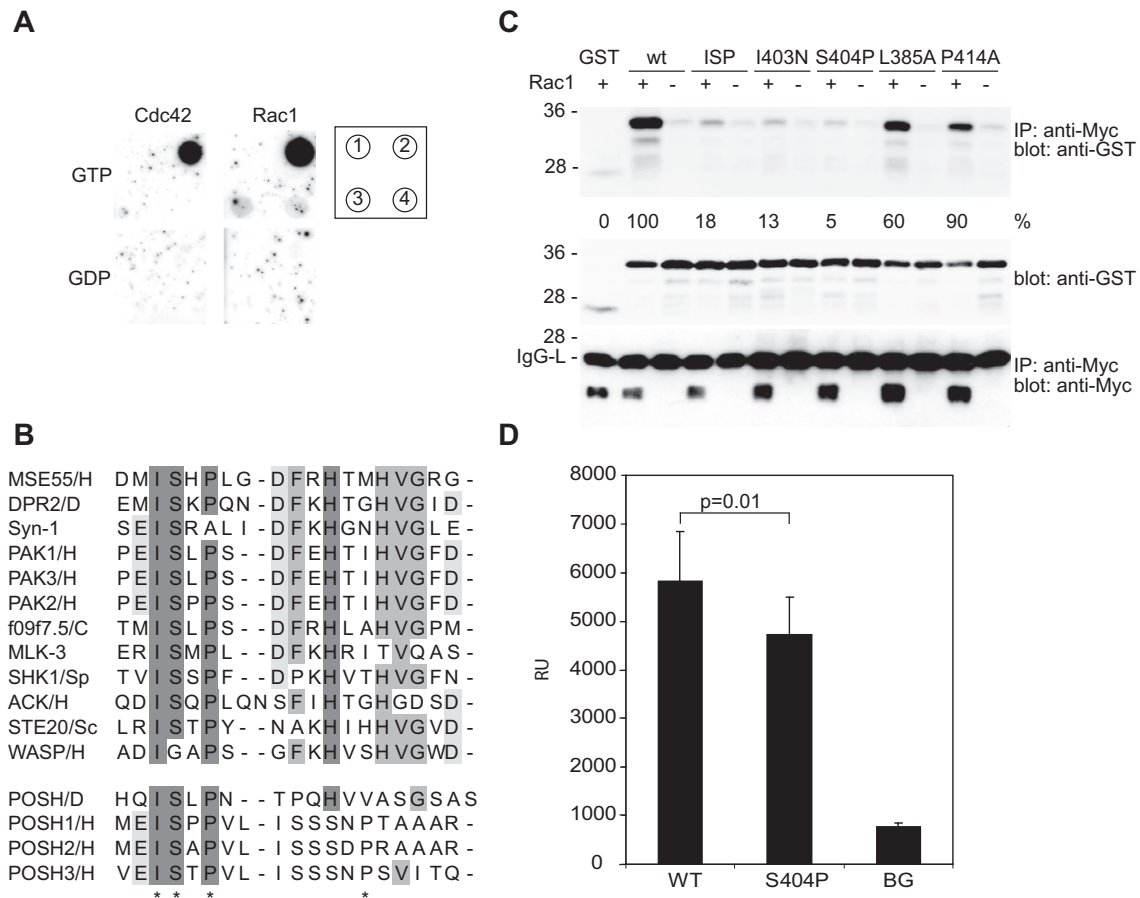
Fig. 3. The partial CRIB domain of POSH2 is required for Rac1 binding. (A) GST (negative control, spot1), GST-PAK2N (positive control, spot 2), GST-POSH1 (AA292–362) (spot 3), and GST-POSH2 (AA369–439) were spotted on nitrocellulose and incubated with either GTP- or GDP-loaded Cdc42 or Rac1 as indicated. Protein quantity and quality has been determined by protein measurement as well as SDS–PAGE. (B) Alignment of proteins containing the CRIB domain that have known Rac or Cdc42 binding activity (taken from [19]), complemented with the comparable region in human POSH1–3 and POSH from *Drosophila melanogaster*. (C) HEK293T cells were co-transfected with myc-tagged Rac1V12 and different POSH2 (AA369–439) wild type and mutant GST fusion constructs. The ISP mutant has all three amino acids are mutated; I403N, S404P, and P406A. The I403N and S404P have single amino acid changes. L385A and P414 are control mutations of conserved amino acids that are on either side of the I-S-X-P motif. Rac1V12 was immunoprecipitated, using anti-Myc, from the cell lysates and the bound POSH2 fragments were analysed by Western blot detection using anti-GST (upper panel). Intensity of the signal was quantified and expressed as percentage of binding as compared to the wild type fragment. Part of the pull down beads was used to control for the presence of Rac1 (lower panel) and part of the lysates was used to determine the amounts of the POSH2 fragments (middle panel). Molecular weight markers are indicated (kDa) (IgG-L is immunoglobulin light chain). (D) JNK activation was measured through an AP-1-luciferase reporter assays in cells transfected with either full length wild type (WT), or S404P mutant POSH2, or without POSH2 (BG). Standard error is indicated.

Mutation of these conserved amino acids in the CRIB domain of p21-activated kinase has been shown to disrupt its interaction with Cdc42 [20]. We therefore generated mutant forms of POSH2 (AA369–439) in which several of these potentially critical amino acids were mutated either alone (I403N and S404P) or combined (I403N/S404P/P406A (ISP)) as well as control mutations L385A and P414 that are conserved amino acids located on either side of the I-S-X-P motif. Because the signal from our GTPase overlay experiments was rather poor we used a different experimental set-up in the mutation analysis. The GTPase interaction was studied by pull down of the POSH GST-fusion proteins by co-transfected dominantly GTP-loaded Myc-tagged Rac (Fig. 3C).

From our experiment it was clear that the I-S-X-P part of the CRIB domain is critical for Rac binding. The amounts of Rac1 protein present on the immunoprecipitation beads were, unfortunately, not identical in all the different samples (Fig. 3C, lower panel). However, the decreased amounts of co-precipitated ISP, I403N, or S404P mutant POSH2 (AA369–439) compared to the wild type fragment can not be explained by reduced amounts of Rac1, since in these samples the Rac1 levels were higher then in the wild type.

3.5. Perturbation of the partial CRIB domain affects JNK activation by POSH2

POSH1 acts as a scaffold for a multiprotein complex that transduces signals from GTP-loaded Rac1 to JNK activation [11,13,21]. Potentially POSH2 would function in a similar way and the anticipated JNK activation should then be perturbed by mutation of the partial CRIB domain. To measure JNK activation we used a luciferase reporter construct consisting of 3 binding sites for the AP-1 transcription factor that drives the expression of luciferase. As shown in Fig. 3D, transfection of wild type POSH2 greatly increased the luciferase expression as compared with the control transfection without POSH2 (BG) indicating that this assay indeed functions as intended.

We generated one of the mutations of the partial CRIB domain (S404P) in the full length POSH2 protein and compared this mutant construct to the wild type protein. Although not very large, the difference between the wild type and mutant construct was significant ($P < 0.01$) indicating that the Rac1 binding through the partial CRIB domain is functionally comparable to the traditional interaction between GTPase and CRIB domain.

Remarkably, there exists another protein with only a partial CRIB domain; the partition-defective protein Par6. Par6 was first identified from the nematode *Caenorhabditis elegans* but subsequently also found in mammals. It is essential for asymmetric cell division and polarized growth and contains a 'semi-CRIB' motif which is necessary, but not sufficient, for binding to Cdc42 and TC10, but not with Rac [22]. The semi-CRIB domain of Par6 (I-S-X-P-X-X-F) [23] consists of a slightly larger part of the CRIB domain then the part we identified in POSH2. In both proteins the C-terminal part of the CRIB domain with the two invariant histidine residues is missing. From the crystal structure of Cdc42 with Par6 it has become apparent that a bordering PDZ (*PSD95/Discs Large/ZO-1*) domain is essential for this interaction. The PDZ domain and the semi-CRIB domain form a continuous structure in which the PDZ domain acts as a structural scaffold for the semi-CRIB domain [23]. In POSH there is no adjacent PDZ domain and it will therefore be of interest to study the possible involvement of other regions of the POSH protein in the interaction with Rac. It is relevant to note in this respect that a major phosphorylation site of Akt in POSH1 is Serine 304 which lies about 22 amino acids C-terminal of the I-S-X-P sequence in the RBD domain. Phosphorylation of this residue was found to decrease the ability of POSH1 to bind Rac [24].

The possible interaction between POSH3 and Rac has not been documented so far. The I-S-X-P sequence is also present in this protein and also the sequence immediately C-terminal is conserved. However the overall sequence homology of POSH3 with POSH1 and POSH2 is not high throughout the RBD fragment. Surprisingly, D. melanogaster DPOSH has been found not to bind to Rac [25] (data presented as 'not shown' in this paper), although the partial CRIB domain is very well conserved according to our alignment. Currently there is no explanation for this, other than a possible lack of sensitivity of the performed assay. The downstream effect of the interaction between POSH1 and Rac is activation of the JNK/SAPK pathway [11,13,24]. This downstream effect is conserved also in Drosophila [25] suggesting a possible involvement of and interaction with Rac.

In conclusion, POSH2 is a ubiquitously expressed E3 ligase of the mammalian family of POSH proteins which has now three members. POSH2 can interact with GTP-loaded Rac using only a partial CRIB domain.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.07.060.

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