UNIVERSITY OF CALIFORNIA, SAN DIEGO

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Identification and Biochemical Characterization of the Phosphotyrosine- and ShcA-binding Protein STS-1

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Chemistry

by

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2017

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2017

DEDICATION

To my Father, who taught me to be curious

To my Mother, who gave me life and taught me love

To my Wife, for her endless support

To my Children, for inspiring me

EPIGRAPH

If I have seen further, it is because I stood on the shoulders of giants.

Sir Isaac Newton

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2HPE	2-histidine phosphoesterase
AEBSF	4-benzenesulfonyl fluoride hydrochloride
AP2	adaptor protein complex 2
Arg	arginine
Asn	asparagine
AT	adenine-thymine
ATP	adenosine triphosphate
BAD	Bcl2-associated agonist of death
BCR	breakpoint cluster region
BCRP	breast cancer resistance protein
CCP	clathrin-coated pit
cDNA	complementary DNA
CME	clathrin-mediated endocytosis
CML	chronic myelogenous leukemia
CRISPR	clustered regularly interspaced short palindromic repeats
D	aspartic acid
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribose nucleic acid
DNA-PK	DNA-dependent protein kinase
DSP	dual-specificity phosphatase
DTT	dithiothreitol
E	glutamic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGR1	early growth response protein 1
EGTA	ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid
ESI	electrospray ionization

FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FGFR	fibroblast growth factor receptor
FKHR	forkhead homolog in rhabdomyosarcoma
G-protein	guanine-nucleotide binding protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GIST	gastrointestinal stromal tumor
Gly	glycine
Grb2	growth factor receptor-bound protein 2
GST	glutathione-*S*-transferase
GTP	guanosine triphosphate
GTPase	guanosine triphosphate phosphatase
H2AX	histone 2AX
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	histidine
I	isoleucine
ICD	intracellular domain
Ig	immunoglobulin
INPP4B	inositol 3,4-bisphosphate 4-phosphatase
IP_3	inositol triphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRS-1	insulin receptor substrate 1
Lys	lysine
M	methionine
MAPK	mitogen-activated protein kinase
MS	mass spectrometry
mTOR	mammalian target of rapamycin

MuSK	muscle, skeletal receptor kinase
N	asparagine
NCBI	National Center for Biotechnology Information
P	proline
PAcP	prostatic acid phosphatase
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PDGFR	platelet-derived growth factor receptor
PDPK1	3-phosphoinositide-dependent protein kinase
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PID	phosphatidylinositide-interacting domain
PIP_2	phosphatidylinositol-bisphosphate
PIP_3	phosphatidylinositol-trisphosphate
PKCΔ	protein kinase C delta
PLC-γ	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase γ-1
PTB	phosphotyrosine-binding domain
PtdIns	phosphatidylinositol
PTK	protein-tyrosine kinase
PTP	protein-tyrosine phosphatase
PVDF	polyvinylidene difluoride
pY	phosphotyrosine
RIP	regulated intramembrane proteolysis
RNA	ribose nucleic acid
RNAi	RNA interference
RPTK	receptor protein-tyrosine kinase
RPTP	receptor protein-tyrosine phosphatase
S	serine
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Ser	serine
SH2	Src homology 2
SH3	Src homology 3
STAT5A	signal transducer and activator of transcription 5a
STS	suppressor of T-cell receptor signaling
T	threonine
TBS	tris-buffered saline
TBST	tris-buffered saline, Tween-20
Thr	threonine
TKI	tyrosine kinase inhibitors
TPA	12-*O*-Tetradecanoylphorbol-13-acetate
Tyr	tyrosine
UBA	ubiquitin-associated
V	valine
VEGFR-1	vascular endothelial growth factor receptor-1
ZAP-70	70 kDa zeta-chain associated protein

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ABSTRACT OF THE DISSERTATION

Identification and Biochemical Characterization of the Phosphotyrosine- and ShcA-binding Protein STS-1

by

Spencer Paul Swarts

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Professor Peter van der Geer, Chair

ShcA is a protein that is recruited to receptor-protein tyrosine kinases, where it is phosphorylated on several tyrosine residues. These phosphotyrosine residues act as binding sites for Grb2. Interestingly, some receptors also bind Grb2 directly, which would suggest a redundant role for ShcA. Recently, we demonstrated that cells expressing receptors engineered to bind ShcA display enhanced rates of cell division compared to cells expressing versions of the receptor designed to bind Grb2 directly. In this dissertation, I describe our efforts

to identify and characterize novel ShcA-binding proteins that may play a role in this phenomenon.

Chapter 1 is an introductory chapter that describes the field of receptor protein-tyrosine kinase signaling.

Chapter 2 describes the use of ShcA-derived phosphopeptides as reagents to purify proteins that bind ShcA in a phosphorylation-dependent manner. This led to the identification of STS-1 as a protein that associates with the phosphorylated Tyr317 region of ShcA. In cell-based experiments, we demonstrate that the association between STS-1 and ShcA is enhanced following growth factor stimulation.

In chapter 3, we demonstrate that STS-1 binds directly to the phosphorylated Tyr317 ShcA-derived peptide. To determine which region of STS-1 directly binds the peptide, two complementary approached were undertaken. In the first, purified STS-1 was subjected to limited proteolysis with thermolysin and the resulting fragments were incubated with the phosphorylated Tyr317 ShcA-derived peptide. A fragment that includes the region of STS-1 with homology to 2H phosphoesterases retained the ability to bind the peptide. In the second approach, mammalian cell lysates expressing STS-1 constructs with point mutations intended to disable individual domains of STS-1 were incubated the phosphorylated Tyr317 ShcA-derived peptide. STS-1 constructs with mutations in the domain with homology to 2HPEs failed to associate with the peptide.

Chapter 4 describes the ability of STS-1 to dephosphorylate (or regulate dephosphorylation) of ShcA. We show that STS-1 can dephosphorylate tyrosine residues of ShcA in vitro. Additionally, we show that depletion of STS-1 results in enhanced ShcA tyrosine phosphorylation and enhanced Akt activation in mammalian cells.

In summary, my results demonstrate that STS-1 binds directly to ShcA in a phosphotyrosine-dependent manner and that STS-1 may also be a physiologically relevant ShcA-phosphatase.

Chapter 1. Receptor protein-tyrosine kinases

1. Introduction

Over the course of evolution, all organisms have gained the ability to respond to stimuli and thus gain an advantage in finding nutrients, avoiding harm, or locating a mate. In order to respond to the external cues, cells utilize cell-surface receptors that bind to - and are activated by - specific molecules that are present in the extracellular milieu. In general, receptor activation results in a series of intracellular protein modifications and protein-protein interactions. These events make it possible for the cell to modulate cell biology, gene transcription and cell morphology.

Unlike unicellular organisms, multicellular eukaryotic organisms must also be able to differentiate and organize their individual cells in order to create a functioning organism. Multicellular eukaryotic organisms begin life as the product of the union of egg and sperm, generating a single cell, the zygote. Amazingly, this cell has the capacity to produce an organism consisting of trillions of differentiated cells that will form the different tissues of the organism. To go from a single cell to a mature organism that carries out all the functions of life, requires an exquisite level of communication amongst the organism's cells.

Among the eukaryotes, metazoans (animals) are unique in that they do not possess a cell wall that limits the movement of their cells. Because of this,

metazoan cells can demonstrate far greater plasticity in their movement. Fungi, eukaryotic relatives of metazoans, do not utilize tyrosine phosphorylation extensively. Metazoans, meanwhile, utilize tyrosine phosphorylation to carry out numerous biological activates including, but not limited to, embryonic development, immune system activation, and tissue repair. These activities require individual cells to migrate to specific locations with appropriate timing. For instance, macrophages sense cytokines that are secreted by damaged cells and migrate to the site of injury, where they phagocytose bacteria and cellular debris. Thus, it appears that tyrosine phosphorylation independently evolved in metazoans as a mechanism that allows their cells to move and organize in ways that are not possible (or necessary) for their closely related cousins. As tyrosine phosphorylation is a critical mechanism for human development and homeostasis, it is not surprising that its dysregulation is also a common source of human maladies, including cancer, autoimmune disorders, and inflammation, among many others. This chapter will focus on the relative importance of protein-tyrosine phosphorylation in relation to human health and disease, with a specific focus on the role of the phosphotyrosine-binding protein ShcA.

2. Receptor protein-tyrosine kinases

Protein-tyrosine kinases (PTKs) are the enzymes responsible for the addition of phosphate to a tyrosine residue in a protein. Specifically, these enzymes catalyze the transfer of the γ-phosphate in adenosine triphosphate

(ATP) to the hydroxyl group present in the side chain of tyrosine residues. Of the 566 protein kinases described in the human genome, 90 are of the PTK variety. Of the 90 PTKs, 58 (64%) are membrane-bound receptor protein-tyrosine kinases (RPTKs) [1]. The 58 RPTKs can be further organized into 20 subfamilies based on sequence similarity and domain architecture [2]. RPTKs are single-pass transmembrane proteins with an extracellular ligand-binding domain and an intracellular kinase domain (Figure 1). Upon ligand binding, most RPTKs will dimerize, often forming homodimers; although some RPTKs form heterodimers with members of the same subfamily. Other RPTKs exist as preformed dimers, the insulin receptor being a classical example [3]. Regardless of their oligimerization state prior to activation, upon cognate ligand binding the kinase domains of RPTKs become activated and allow the receptors to engage in autophosphorylation, which involves the *trans* phosphorylation of tyrosine residues in the juxtaposed receptor [4] (Figure 3). Phosphorylation of certain tyrosine residues within the kinase domain enhances kinase activity [5]. Other phosphotyrosine residues in the RPTK act as binding sites and recruit proteins containing SH2 or PTB domains and are essential in communicating ligand binding into a biochemical change within the cell [6,7].

RPTK expression demonstrates considerable variability with some RPTKs performing vital functions in a variety of cell types and others showing more limited expression and function. For instance, the insulin receptor is present on many cell types including muscle, liver, pancreatic β-cells, neurons,

bone, retina and macrophages. Activation of the insulin receptor regulates glucose transport, glycogen biosynthesis, and mitogenesis. Other RPTKs display a much more specialized role, such as the MuSK receptor, which mediates clustering of acetylcholine receptors at the neuromuscular junction [8,9].

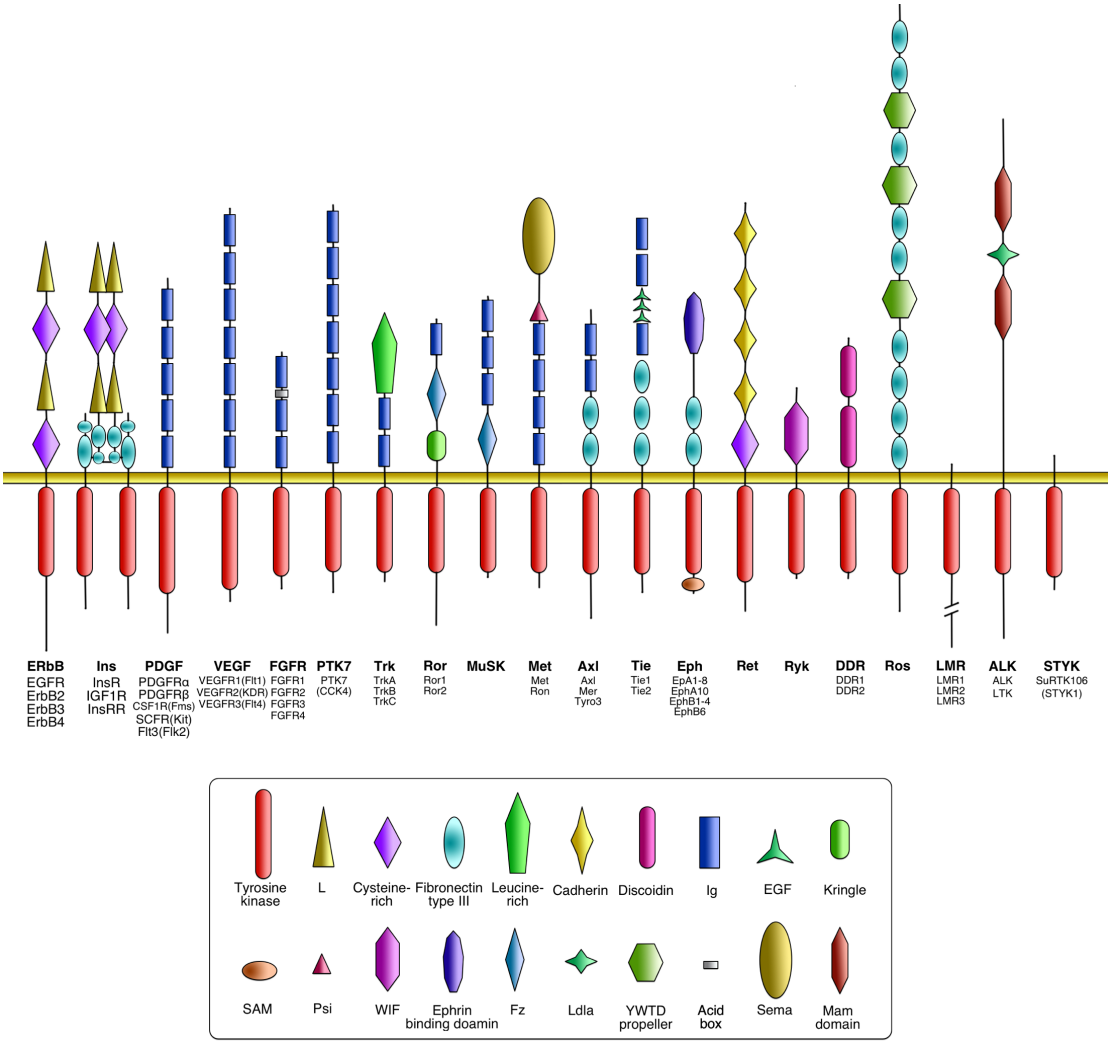

Figure 1.1 Receptor protein-tyrosine kinase subfamilies
The human RPTKs can be grouped into 20 subfamilies according to domain architecture and sequence similarity. Here, the domain architecture of each subfamily is depicted according to the key. Receptor family names are indicated in bold, with individual members listed below if more than one member exists.

RPTK signaling can be paradoxical, often activating overlapping groups of proteins to obtain distinct biochemical outcomes. For example, stimulation of PC12 cells with either epidermal growth factor (EGF) or nerve growth factor (NGF) induces activation of mitogen-activated protein kinase (MAPK). Interestingly though, in these cells EGF stimulation leads to cellular proliferation whereas NGF stimulation leads differentiation and neurite outgrowth [10]. The major difference appears that EGF induces only transient MAPK activation, while NGF stimulation leads to sustained MAPK activation [11]. Overexpression of the EGF receptor in these cells lead to them to differentiate upon EGF stimulation, likely due to a shift from transient to sustained MAPK activation. It is proposed that the shift in signaling outcome is due to positive and negative feedback mechanisms within the cell [12].

The intricacy of RPTK signaling is further illustrated by the fact that multiple ligands can activate the same receptor and that a single receptor can bind multiple ligands. In otherwise identical cells, the activation of a particular receptor with different ligands can lead to distinct signaling outcomes. Activation of the fibroblast growth factor receptors (FGFRs) is perhaps the best example of this phenomenon. FGFR signaling during embryonic development regulates cell proliferation, migration and differentiation. In adult tissues, FGFR signaling functions in tissue repair and response to injury, again by regulating the proliferation, migration and differentiation of many cells types. Alternative splicing of the four *FGFR* genes results in the production of over 48 different

isoforms. Additionally, humans possess 22 genes that encode more than 30 different FGF proteins [13]. These proteins display a complex web of activity, where a particular FGF may bind all FGFRs, a particular subset of FGFRs, or none at all [14].

2.1 RPTK activation

Classically, RPTKs have been studied in the context of their activation by growth factors at the plasma membrane and the cascade of events which take place thereafter. As mentioned, following ligand binding, RPTKs engage in *trans* autophosphorylation of intracellular tyrosine residues of the juxtaposed receptor. Tyrosine phosphorylation of the receptor causes an increase in kinase activity and also generates binding sites for proteins with Src homology-2 (SH2) or phosphotyrosine-binding (PTB) domains. These events initiate the signaling cascade that occurs upon RPTK activation in response to ligand stimulation [15–19].

Interestingly, the majority of RPTK families have been detected in the nucleus [20,21]. Following ligand-induced activation, RPTKs can be internalized and translocated to the nucleus. Appearing as either holoreceptors or in a truncated form, these nuclear RPTKs appear to play a role in cell proliferation and invasion, DNA damage and repair as well as transcriptional regulation [22–25].

2.2 Nuclear RPTK signaling

It is proposed that RPTKs can translocate – intact or fragmented – to the nucleus through a variety of mechanisms including regulated-intermembrane proteolysis (RIPing), retrograde transport and possibly other undescribed mechanisms. 11 of the 20 RPTK subfamilies have been detected in the nucleus, where their activity is often distinct from their activity the plasma membrane. Here, we provide a brief discussion of the role of RPTKs in the nucleus.

Despite being detected in the nucleus for some time, the first functional reports of nuclear Epidermal Growth Factor Receptor (EGFR) came in 2001, when researchers demonstrated that nuclear EGFR functions as a transcriptional regulator by binding to the AT-rich response sequence of the cyclin D1 promoter and enhancing cyclin D1 expression [26]. Following EGF stimulation, full length EGFR translocates to the nucleus via retrograde transport. There, it is proposed that EGFR associates with RNA helicase A [27] and MUC1 [28] to bind DNA and regulate the expression of genes related to normal physiological and pathological processes including cellular proliferation, inflammation and drug resistance. In the nucleus, EGFR may also act as a tyrosine kinase, as its expression has been correlated with phosphorylation of several nuclear proteins including the proliferative cell nuclear antigen (PCNA) [29] and DNA-dependent protein kinase (DNA-PK) [30]. More recently, expression of genes involved in tumorigenesis were found to be regulated by nuclear EGFR including c-Myc [23], thymidylate synthase [31], and breast cancer resistance protein (BCRP/ABCG2) [32]. Other reports show that EGFR

expression is important for the cell's DNA repair [33], a finding that is consistent with the notion that nuclear EGFR is important for the DNA damage response and DNA damage repair.

ErbB-4 (a member of the EGFR family) is detected in the nucleus following stimulation with 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) or heregulin. The ErbB-4 receptor translocates to the nucleus via the RIP pathway, which is initiated by cleavage of the cell-surface receptor by tumor necrosis factor-α-converting enzyme (TACE). This results in a fragment of the receptor that includes the intracellular and transmembrane region. γ-secretase cleavage within the transmembrane region then releases the intracellular domain (ICD) into the cytosol, leading to translocation of the ICD to the nucleus [34]. In the nucleus, the ErbB-4 ICD associates with STAT5A [35] and Eto-2 to regulate gene expression, including that of β-casein and Eto-2. Like all other members of the EGFR family, full length ErbB-4 is also detected in the nucleus and appears to have distinct activity compared to the ErbB-4 ICD.

Members of the EGFR family are frequently overexpressed or demonstrate constitutive activation in tumors of epithelial origin [36]. To combat this, a variety of anticancer therapies have been developed to target the EGFR and ERbB-2 receptor, including the monoclonal antibodies cetuximab and trastuzumab. Interestingly though, it appears that translocation of the EGFR is critical for it to carry out its pleiotropic effects. For instance, EGF-EGFR cell surface complexes stabilized by concanavalin A are unable to induce DNA

synthesis, despite their ability to induce phosphorylation of cellular proteins and enhance RNA synthesis [37]. In summary, RPTKs emanate signals from diverse cellular locations, not simply at the plasma membrane.

2.3 Ligand-induced RPTK activation

A critical aspect of kinase regulation is the conservation of low basal activity in the absence of an activating stimulus. This is particularly critical, considering that RPTKs are often localized together in a way that favors their oligomerization and subsequent activation, even in the absence of stimulation. Additionally, aberrant signaling from RPTKs can cause a wide variety of ailments in humans, such as cancer, diabetes and other autoimmune diseases. Thus, RPTKs have evolved a variety of regulatory mechanisms to prevent inappropriate activation. RPTKs exist in equilibrium between active and inactive states. Ligand binding shifts the equilibrium toward the active state, which disfavors their activation in the absence of a ligand. RPTKs may also cluster on the cell surface upon activation, leading to a strong, sustained signal. By limiting the expression of a receptor, a cell can prevent significant clustering and the enhanced signaling that would follow.

Several RPTKs exist as monomers on the cell surface, including the platelet-derived growth factor receptor (PDGFR), ErbB, Kit, and Trk subfamilies. These receptors dimerize upon ligand binding, leading to their activation (Figure 2a). Other RPTKs exist as preformed dimers in the absence of stimuli, yet are

not activated. For instance, the insulin receptor subfamily, whose members exist as disulfide-linked dimers in both the inactive and active state [38] (Figure 2b). Ligand binding to members of the insulin receptor subfamily induces conformational changes that lead to its activation, however the exact details are not yet resolved. Other RPTKs exist in equilibrium between a monomeric and dimeric form in the absence of stimulation. For instance, a variety of methods have demonstrated that the EGFR exists in equilibrium between a monomeric and dimeric state on the cell surface [39,40] (Figure 2C). Although it has been proposed that ligand binding induces the formation of higher order oligomers, which leads to activation of the EGFR [41], a detailed mechanism of EGFR activation is also lacking. The Eph receptor family displays yet another mechanism of RPTK activation (Figure 2d). The Eph receptors bind to Ephrins (ligands) which are cell-surface proteins that the may be encountered by Eph receptors on an adjacent cell. Eph receptors exist in equilibrium between monomeric and higher order oligomers in the absence of ligand. Upon encountering Ephrins, it is thought that the Eph receptors cluster through interaction with other Eph Receptor-Ephrin complexes. This results in “forward signaling” in the Eph receptor-containing cells and “reverse signaling” in the Ephrin-containing cells [42].

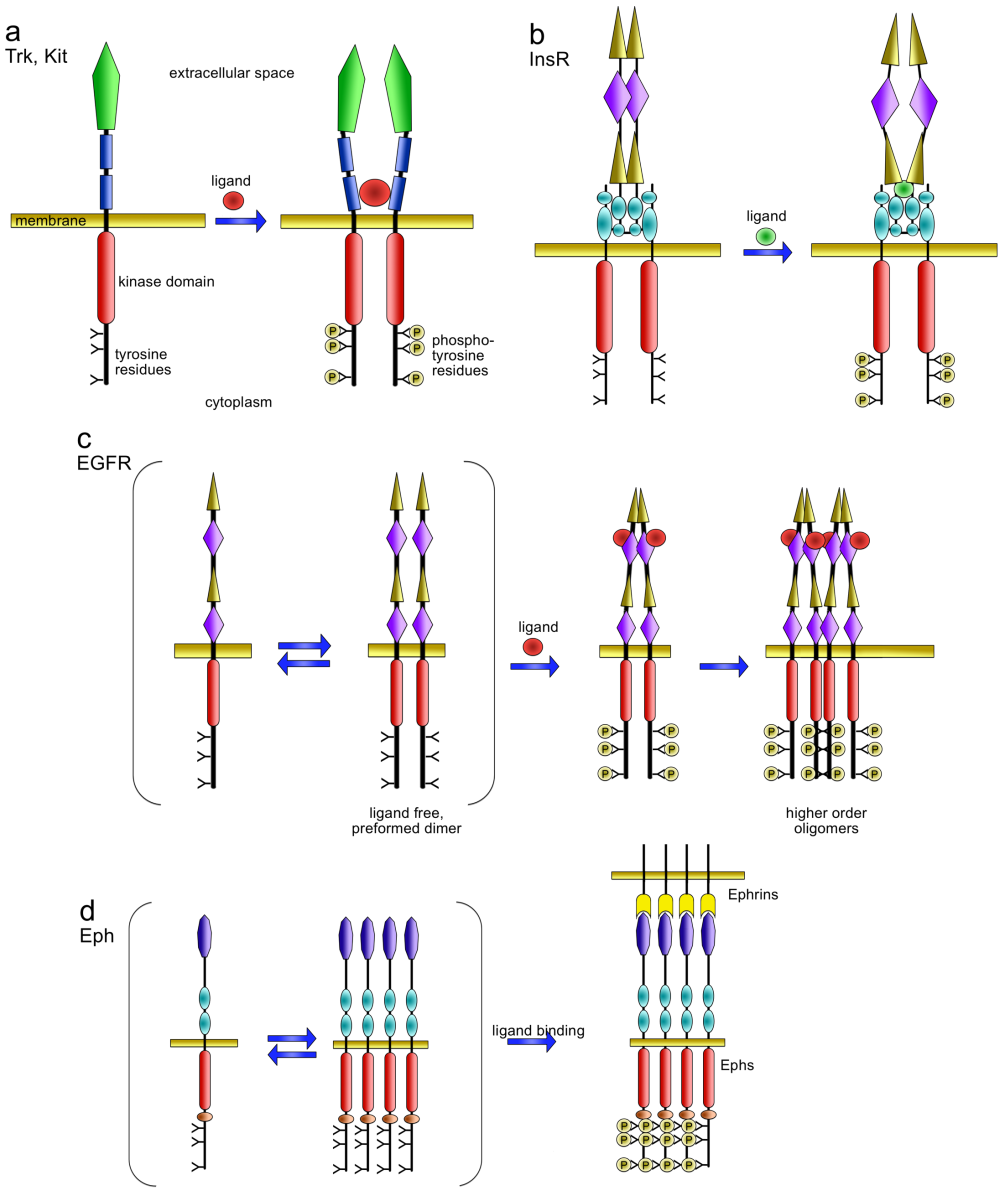

Figure 1.2 Mechanisms of RPTK activation
(a) Canonical mechanism of RPTK activation - depicted here by the NGFR receptor - where receptors exist in monomeric form on the cell surface in the absence of ligand. Ligand binding induces receptor dimerization and tyrosine phosphorylation in trans. (b) The insulin receptor (InsR) is a covalently bound dimer; ligand binding induces conformational changes that activate the kinase domain, leading to phosphorylation of intracellular tyrosine residues. (c) The EGFR exists in equilibrium between monomeric and dimeric forms in the absence of ligand. Ligand binding induces conformational changes that lead to phosphorylation and the formation of higher order oligomers. (d) Inactive Eph receptors form oligomers in the absence of ligand. Ephrins are transmembrane proteins that act as ligands for Eph receptors. When Eph receptors encounter Ephrins on an adjacent cell, the Ephs become activated.

In the absence of a cognate ligand, most RPTKs are kept inactive by the presence of an “activation loop” which bind to the active site of the kinase and/or keeps the kinase in an inactive conformation. This mechanism ultimately inhibits substrate and co-factor binding, preventing activation. For instance, the insulin receptor kinase domain contains three tyrosines in the activation loop, (Tyr 1158, Tyr 1162, Tyr 1163) which must be phosphorylated for activation. In the inactive state Tyr 1162 is positioned in the active site, not unlike a peptide substrate, blocking access to substrate. Furthermore, the presence of the activation loop distorts the active site and prevents ATP and magnesium binding [43]. Crystal structures of the active insulin receptor show the three phosphorylated tyrosine residues in an extended conformation – no longer positioned within the active site – where they determine substrate specificity of the kinase by forming part of the substrate-binding site.

Interestingly, the activation loop can also play a role in generating “kinase-defective” receptors. Although considered “kinase-defective”, the vascular endothelial growth factor receptor-1 (VEGFR-1) is required for normal development and angiogenesis, as demonstrated by *VEGFR-1*$^{-/-}$ mice, which die early embryonic deaths due to abnormal overgrowth of endothelial cells [44,45]. The VEGFR-1 acts as a decoy receptor by binding VEGF and preventing it from activating VEGFR-2 and thus negatively regulates growth [46,47]. Analysis of the activation loop in the VEGFR-1 reveals that a highly conserved aspartic acid residue is replaced by asparagine (Asn1050).

Interestingly, mutation of this residue to aspartic acid establishes kinase activity, allowing the receptor to engage in *trans* phosphorylation upon ligand stimulation.

While maintaining a low level of basal activity at the plasma membrane, RPTKs are rapidly activated by ligand binding. Many RPTKs exist as monomers on the plasma membrane surface and oligomerize upon ligand binding, followed by the *in trans* phosphorylation of intracellular tyrosine residues in the adjacent receptor. Other RPTKs exist as oligomers in the absence of ligand and undergo structural changes that induce intracellular tyrosine phosphorylation when activated. Tyrosine phosphorylation of certain residues enhances the catalytic activity of the receptor, while other phosphotyrosine residues act as binding sites for proteins containing SH2 or PTB domains.

3. RPTK-binding proteins

Following RPTK activation and subsequent phosphorylation of intracellular tyrosine residues, a multitude of proteins are recruited to the plasma membrane where they form signaling complexes with an activated RPTK. The most immediately relevant proteins are those containing SH2 or PTB domains; many of these proteins bind directly to RPTKs in a phosphotyrosine-dependent manner. Moreover, many are either a substrate for the RPTK or recruit additional proteins to serve as substrates for tyrosine phosphorylation. As mentioned previously, there is often overlap in the pathways activated by

RPTKs, largely because of considerable commonality in the proteins that are recruited directly to them. Frequently activated pathways include Ras-Raf-Mek-Erk, phosphoinositide-3-kinase (PI3K)-Akt and phospholipase-C-gamma (PLC-γ) signaling.

3.1 Phosphotyrosine-dependent binding

SH2 and PTB domains are modular portions of proteins that confer the ability to bind in a phosphotyrosine-dependent manner. SH2 and PTB domains bind phosphotyrosine primarily in the context of the adjacent amino acids and connect diverse groups of proteins like kinases, phosphatases, adaptors, and ubiquitin ligases to activated RPTKs [48] (Figure 3). SH2 and PTB domains are known to bind a wide variety of other tyrosine-phosphorylated proteins, however, here, we will focus primarily on their interactions with RPTKs. Although it will not be discussed further here, the C2 domain of PKCδ is among a small group of non-SH2/PTB proteins that also display phosphotyrosine-dependent binding [49].

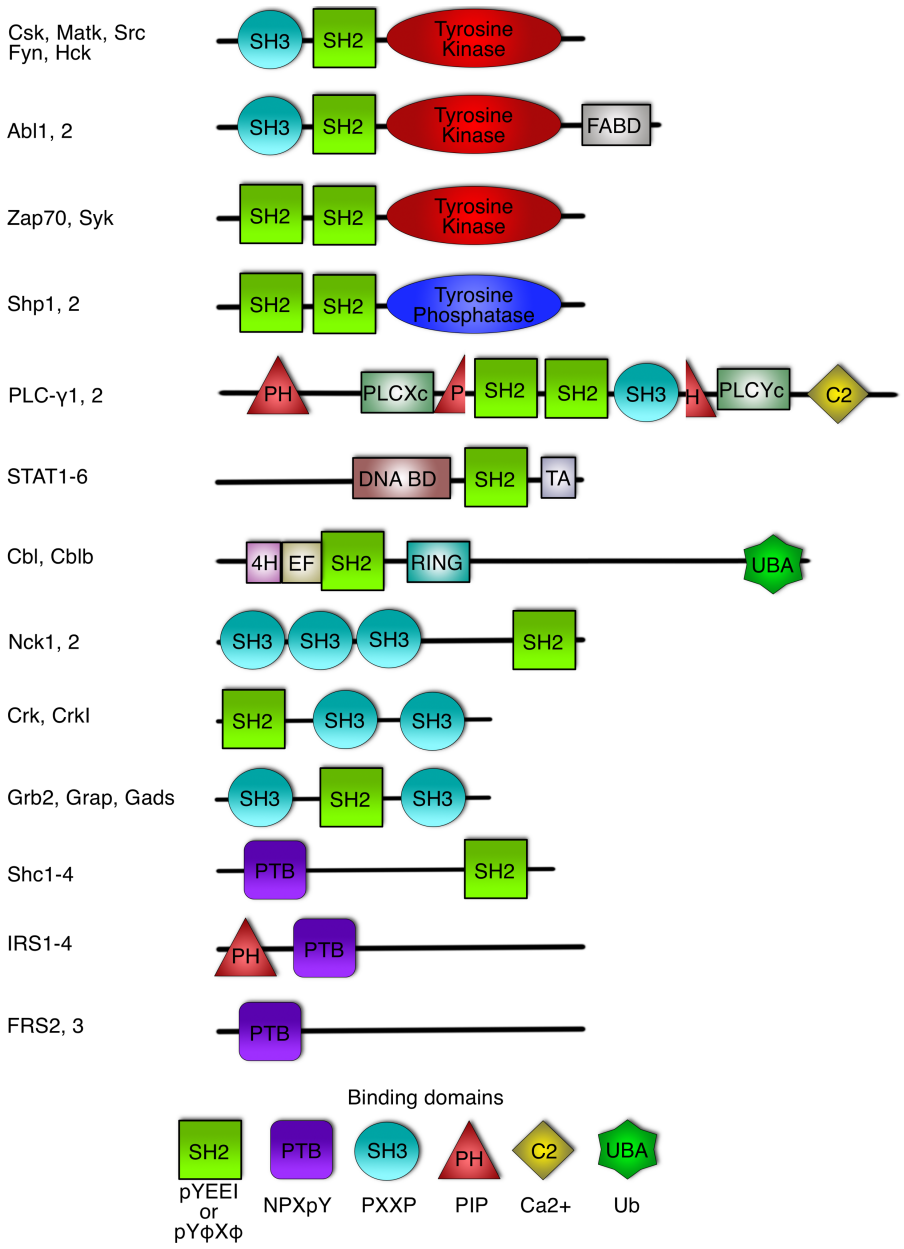

Figure 1.3 Selected SH2- and PTB-containing proteins
Top, the domain architecture of selected proteins that contain SH2 and/or PTB domains and also bind directly to RPTKs. These proteins contain a diverse set of catalytic and non-catalytic binding domains. Bottom, the binding motifs of selected domains are shown below each individual domain. SH2 (Src Homology-2) domains bind phosphotyrosine in the context of the N-terminal residues, preferring one of two motifs where pY represents phosphotyrosine, ϕ is a hydrophobic amino acid and X is any amino acid. PTB (Phosphotyrosine-binding) domains bind phosphotyrosine residues in the context of the C-terminal residues, preferring the motif NPXpY, where X is any amino acid and pY represents phosphotyrosine. SH3 (Src Homology-3) domains bind polyproline motifs. PH (Pleckstin Homology) domains bind phosphotadyl inositol phosphates (e.g. PIP, PIP_2, PIP_3). C2 domains bind calcium (Ca^{2+}), while C1 domains bind diacylglycaerol (DAG) and UBA (Ubiquitin-association) domains bind ubiquitin (Ub).

3.2 SH2 domains

In humans, there exists 120 SH2 domains that appear in 110 unique proteins [50]. Significantly, it appears that the expansion and diversification of SH2 domain-containing proteins occurred concomitantly with the increasing complexity of metazoans [51,52]. This supports the notion that tyrosine phosphorylation evolved in metazoans as a mechanism for cellular organization and communication. SH2 domains bind to a variety of phosphotyrosine-containing proteins, many of which are RPTKs. SH2 domains consist of an antiparallel β-sheet (comprised of three or four β-strands) sandwiched by two α-helices (Figure 4). An invariant arginine residue within SH2 domains forms part of a positively charged pocket to which the phosphotyrosine residue binds [53]. The phosphorylated ligand straddles the β-sheet, with residues immediately carboxy-terminal to the phosphotyrosine residue interacting with the SH2 domain. For example, the SH2 domain of Lck binds the motif pYEEV in this fashion (Figure 4a). Aspartic acid residues in the peptide interact with the β-sheet residues, while the valine residue (pY+3) sits in a hydrophobic pocket near C-terminal α-helix. For the SH2 domain of v-Src, almost identical binding is seen with the peptide motif pYEEI (Figure 4b). The ShcA SH2 domain also shows important hydrophobic contacts with the pY+3 amino acid, as shown in figure 4c.

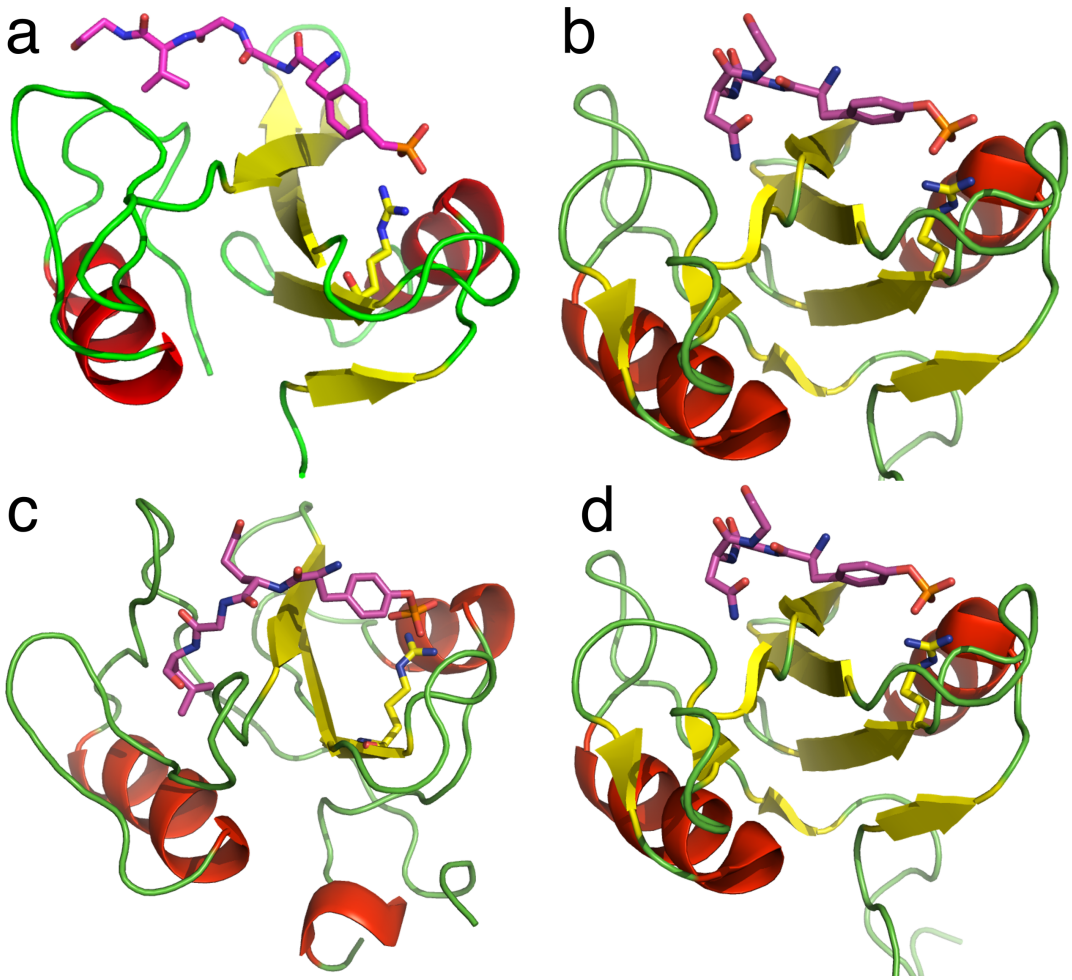

Figure 1.4 SH2 domains bind phosphotyrosine and adjacent carboxy-terminal amino acids. The solved structures of most SH2 domains display a conserved structure, consisting of a β-sheet sandwiched by two α-helices. The binding of a tyrosine-phosphorylated protein to an SH2 domain is primarily dependent on the residues immediately C-terminal to the phosphotyrosine residue. Depicted here are the SH2 domains of **(a)** Lck (PDB ID 1CWD) **(b)** v-Src (PDB ID 1SPS) **(c)** ShcA (PDB ID 1TCE) **(d)** GRB2 (PDB ID 1QG1) bound to tyrosine-phosphorylated peptides. Binding occurs over a conserved region of the SH2 domain, with minor variations. For clarity, only the phosphotyrosine (pY) and pY+3 side chains are shown, except for **(d)**, which shows pY and pY+2 side chains. An invariant arginine, which extends from a central β-strand of the SH2 domains, coordinates phosphate binding. Portions of the peptides not directly interacting with the SH2 domains have also been omitted.

Other SH2 domains, such as that of Grb2, display a different mode of ligand binding. The Grb2 SH2 domain preferentially binds pYXNX motif (where X is any of the natural 20 amino acids) [54]. An essential tryptophan residue

within the SH2 domain mediates interaction with the peptide asparagine, which must occur at the pY+2 position for binding to occur (Figure 4d).

Several SH2 domains bind their ligands in a non-canonical fashion. c-Cbl, an E3 ubiquitin ligase, contains an SH2 domain that is quite divergent in primary sequence when compared to other SH2 domains [55]. The SH2 domain of c-Cbl prefers the motif (N/D)XpY(S/T)XXP [56,57], which unlike other SH2 domains, involves binding residues amino-terminal to the phosphotyrosine. In general though, it is the primary sequence that is carboxy-terminal to a phosphotyrosine residue that determines whether a given SH2 domain-containing effector protein will be recruited.

3.3 PTB domains

The human proteome includes 60 phosphotyrosine-binding (PTB) domain-containing proteins. The docking protein ShcA became the founding member of the group when it was shown a conserved, amino-terminal region of this protein (ShcA was then known to contain a carboxy-terminal SH2 domain) associated with the EGF receptor in a tyrosine phosphorylation-dependent manner [58–60]. Unlike SH2 domains, a variety of PTB domains have been shown to exhibit tyrosine phosphorylation-independent binding. In fact, a global analysis of PTB domains has revealed three distinct classes. Uhlik and others [61] classified PTB domains as either phosphotyrosine-dependent ShcA-like, phosphotyrosine-dependent IRS-like, or phosphotyrosine-independent Dab-like

based on structural, functional and evolutionary data. Despite these considerable differences, all PTB domains appear to bind the NPX(p)Y motif.

The PTB domain of ShcA contains a positively charged pocket that interacts with the phosphotyrosine residue. Basic residues form this pocket and include two arginines and one lysine (Arg67, Arg175, and Lys169 in ShcA, Figure 5a). IRS-like PTB domains also contain a positively charged pocket for phosphotyrosine binding, typically composed of two arginine residues (Arg212 and Arg227 in IRS-1, Figure 5b). Not surprisingly, Dab-like PTB domains have a less basic and shallower binding pocket which is consistent with their ability to engage in phosphotyrosine-independent binding. In the tyrosine-binding pocket of Dab2, for example, His135 and Gly131 form van der Waals contacts and hydrogen bonds, respectively, with the tyrosine residue (Figure 5c). Other residues in a PTB domain make various hydrophobic contacts and hydrogen bonds with the amino acids amino-terminal to the tyrosine residue and provide specificity to binding [62].

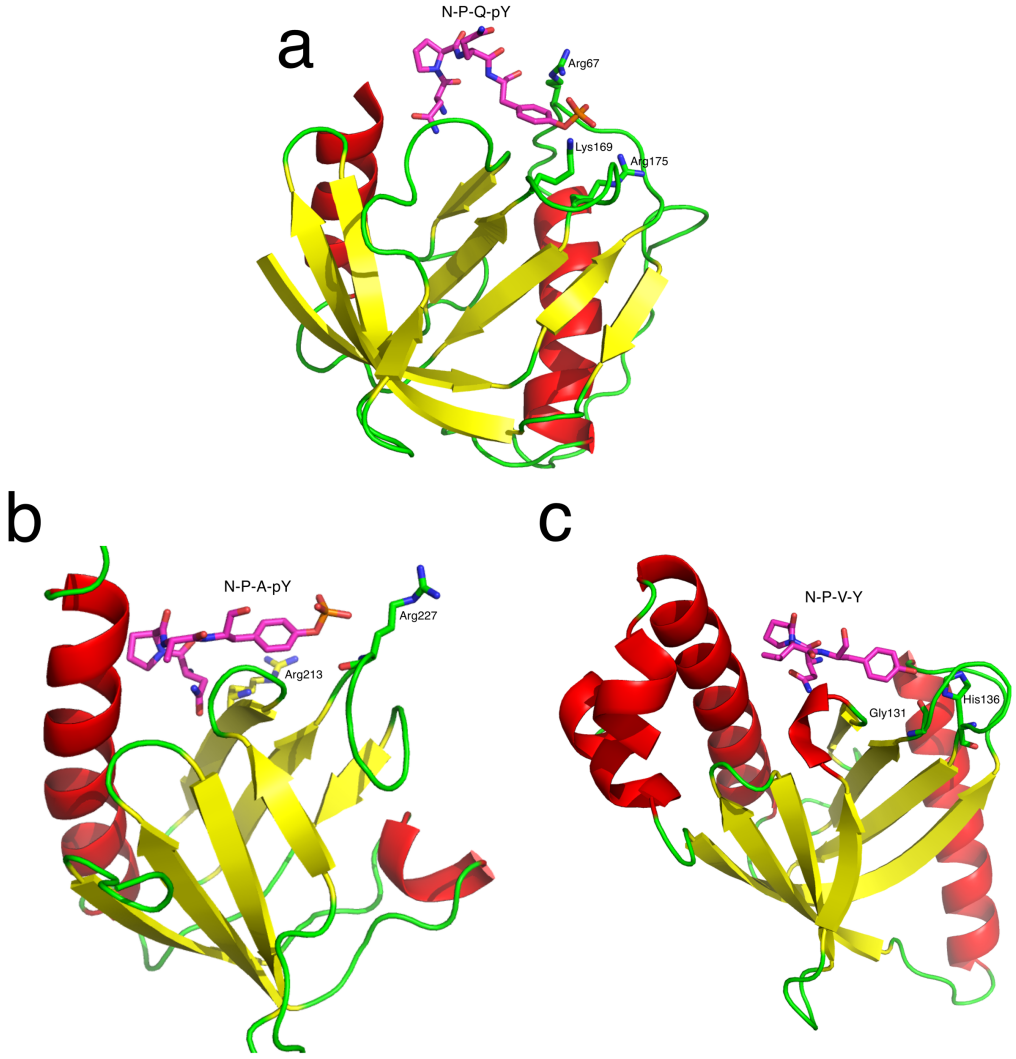

Figure 1.5 PTB domains bind to tyrosine-phosphorylated and unphosphorylated peptides by recognizing residues amino-terminal to the tyrosine residue

All PTB domains bind the motif NPX(p)Y, where N in asparagine, P is proline, X is any of 20 natural amino acids, pY is phosphotyrosine, and Y is tyrosine. **(a)** The PTB domain of ShcA (PDB ID 1SHC) binds in a phosphorylation-dependent manner to the motif NPQpY. Arg67, Lys169, and Arg175 create a positively charged pocket to coordinate phosphotyrosine binding. **(b)** The PTB domain of IRS-1 (PDB ID 1IRS) also binds in a phosphorylation-dependent manner, preferring the motif NPApY. Here, Arg213 and Arg227 comprise the phosphotyrosine-binding pocket. **(c)** The PTB domain of Dab2 (PDB ID 1NTV) binds its ligand in a phosphorylation-independent manner, shown here bound to the motif NPVY. To accommodate the tyrosine residue, His136 forms van der Waals contacts and the carbonyl group of Gly131 forms hydrogen bonds. This figure depicts the three modes of PTB-peptide binding.

Interestingly, proteins containing a PTB domain appear to lack catalytic activity. Although the evolutionary reason for this is unknown, proteins with PTB domains often contain additional domains that mediate protein-protein interactions (Figure 3), as well as tyrosine residues that are phosphorylated upon RPTK binding. These features are essential for PTB domain-containing proteins to propagate signaling from an activated RPTK and will be discussed below.

Many PTB domains can also bind phospholipid acidic head groups at region distinct from the region that binds the NPX(p)Y motif. Additionally, PTB domains are also referred to as phosphotyrosine-interacting domains (PIDs) [63].

4. Proteins activated by RPTKs

As mentioned, ligand-induced RPTK activation results in autophopshorylation of intracellular tyrosine residues. These modifications release intramolecular inhibitory interactions and also generate binding sites for SH2- and PTB-domain containing proteins. In healthy cells, signaling from RPTKs is linked to cell growth, survival, division and differentiation. However, much of what has been studied in regards to RPTKs and their downstream effectors is related to pathologies such as cancer. The following discussion will focus on the behavior of proteins downstream of RPTKs and some of their relationships to such pathologies.

4.1 Ras-Raf-Mek-Erk Signaling

Stimulation of many RPTKs results in the activation of Ras-Raf-Mek-Erk (Ras-Erk) signaling, which induces cellular behaviors, such as, growth, differentiation and division. The Ras proteins are a part of a large family of small G-proteins that are activated as a consequence of their interaction with guanine-nucleotide exchange factors (GEFs). GEFs act by binding G proteins and stimulating the release of guanosine diphosphate (GDP). Guanosine triphosphate (GTP) then preferentially binds the G protein due to the tenfold higher intracellular concentration of GTP to GDP. As mentioned, the activation of G proteins is a critical step in signal transduction in healthy cells. Yet these proteins can become constitutively activated due to mutation, leading to sustained mitogenic signaling. Activating mutations in three *Ras* family members, *H-Ras*, *K-Ras* and *N-Ras*, are found in about 25% of all tumors and up to 90% in pancreatic tumors [64]. Activating mutations in these Ras proteins are accounted for by alterations in only three codons, including codons 12, 13 and 61, forcing the G-protein in the “on”, GTP-bound state [65].

The growth-factor-receptor-bound-protein-2 (Grb2)/Son-of-Sevenless (SOS) complex is perhaps the best-characterized module that links RPTKs to Ras activation (Figure 6a). Grb2 binds RPTKs in a tyrosine-phosphorylation-dependent manner via a central SH2 domain. The N-terminal SH3 domain in Grb2 constitutively binds SOS [66], which is a GEF. SOS, in turn, induces structural changes in Ras to promote the release of GDP, which is quickly

replaced by GTP. GTP-Ras then activates the serine kinase Raf through a direct association that induces a conformational change and allows Raf to phosphorylate its various substrates [67], most notably the dual-specificity kinases Mek1 and Mek2 [68].

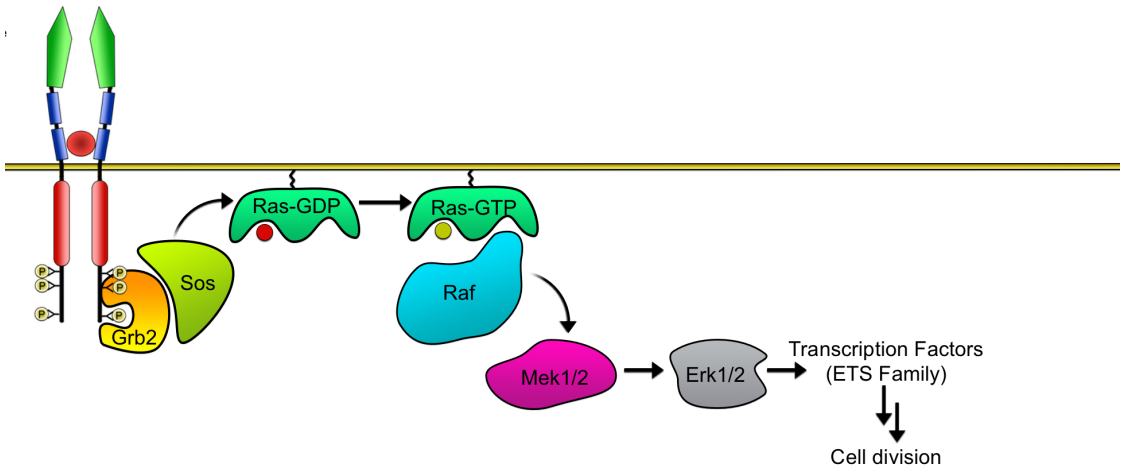

Figure 1.6 Ras-Erk Activation
Grb2 binds many RPTKs in a phosphotyrosine-dependent manner. Sos, which is constitutively bound to Grb2, catalyzes Ras to exchange GDP for GTP. Ras-GTP then binds and activates Raf, which phosphorylates and activates Mek1/2. Phosphorylated Mek1/2 then phosphorylates Erk1/2 on threonine and tyrosine. Phosphorylated Mek1/2 can then phosphorylate numerous substrates in the cytosol and nucleus.

Mek1 and Mek2 are potent activators of the mitogen-activated-protein kinases (MAPK) Erk1 and Erk2 (Erk1/2). Erk1/2 are, in turn, critical mediators of cell cycle progression. Erk1/2 are serine kinases whose targets include a variety of cytosolic and nuclear targets, with their activity on transcription factors being the most-well detailed process in which they participate. Erk1/2 phosphorylates ETS-family transcription factors, such as Elk1. Elk1 is then able to form a complex with serum response factor (SRF) at serum response element

(SRE) sites on DNA, thereby regulating the expression of genes such as *Fos* [69]. Additionally, Erk1/2 phosphorylate c-Jun to generate the AP1 transcription factor, which is composed of Fos/c-Jun heterodimers [70]. Ultimately, Erk1/2 activation of AP1 and other transcription factors induces the expression of key cell-cycle regulators, allowing cells to progress through G1 of the cell cycle.

4.2 Phosphatidylinositol 3-kinase/Akt

The activation of phosphatidylinositol 3-kinase (PI3K) and Akt work in concert to promote cellular division in both normal and cancerous cells. These proteins are activated as a consequence of PI3K recruitment to activated RPTKs.

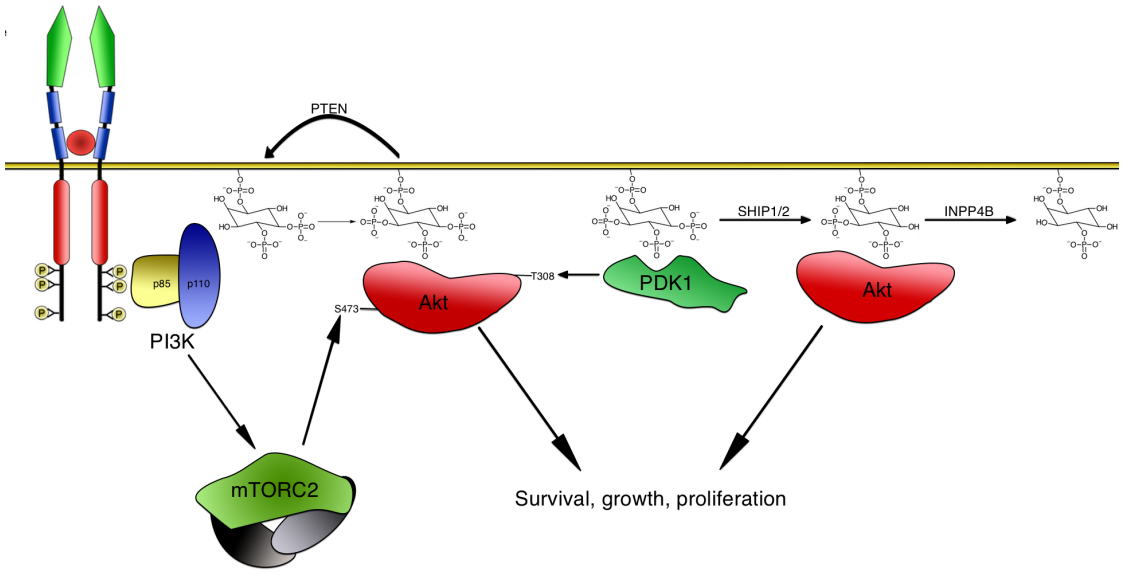

Figure 1.7 PI3K-Akt activation
Upon activation, many RPTKs also bind PI3K where PI3K phosphorylates the 3' position of PIP_2, yielding PIP_3. PI3K also induces activation of the mTORC2 complex, which results in the phosphorylation of Akt at S473. The presence of PIP_3 and additional T306 phosphorylation leads Akt to phosphorylate numerous proteins involved in survival, growth, and proliferation.

The PI3K family of enzymes catalyze the phosphorylation of the 3-position of phosphatidylinositols (PtdIns). Several classes of PI3K enzymes exist, with the most well-understood belonging to class IA. Class IA PI3K enzymes are recruited to activated RPTKs by an SH2 domain which binds the tyrosine-phosphorylated YXXM motif in the receptor. PI3K translocation to the plasma membrane is sufficient for its activation and results in the phosphorylation of PtdIns(4,5)P_2 (PIP_2) which generates PtdIns(3,4,5)P_3 (PIP_3)[71] (Figure 6b).

The Akt proteins (Akt1, Akt2, Akt3) are a family of serine/threonine kinases. These proteins, the cellular homologs of the retroviral oncogene v-Akt, bind PIP_3 via a PH domain. For Akt to become maximally activated it must be phosphorylated at Thr308 and Ser473 by PDPK1 and mTORC2, respectively [72,73]. Translocation to the plasma membrane (via interaction with PIP_3) and subsequent phosphorylation of Akt activates kinase activity, allowing it to phosphorylate a wide-array of signaling proteins, such as mTOR, BAD, and Forkhead family transcription factors. These proteins then act to affect gene transcription in ways that inhibit apoptosis and enhance cellular survival, proliferation.

Akt directly phosphorylates several members of the apoptotic machinery, including BAD, a member of the BCL2 family of proteins that promotes cell death by forming heterodimers with the survival factor BCL-X_L, rendering it non-functional. Akt phosphorylation of BAD prevents formation of these

heterodimers, thus restoring the anti-apoptotic ability of BCL-X_L [74]. Akt-mediated phosphorylation of the pro-death protease, caspsase-9, inhibits its catalytic activity [75], again inhibiting apoptosis. Additionally, Akt phosphorylates FKHR, a transcription factor that translocates to the nucleus and activates several pro-apoptotic genes. Akt phosphorylation of FKHR prevents FKHR nuclear translocation [76].

PIP_3 is degraded by lipid phosphatases, such as PTEN, SHIP1 and SHIP2. Whereas SHIP1 and SHIP2 remove phosphate from the 5-position to generate PtdIns(3,4)P_2, PTEN dephosphorylates PIP_3 at the 3-postion to regenerate PIP_2. Interestingly, PtdIns(3,4)P_2 retains the ability to recruit PH-domain containing proteins, such as Akt. INPP4B dephosphorylates PtdIns(3,4)P_2 at the 4-position, yielding PtdIns(3)P [77]. Thus, the activity of PTEN and INPP4B is primarily responsible for controlling the mitogenic effects of PIP_3, as evidenced by the fact that *Pten*- and *INPP4B*-knockout mice are strongly predisposed to oncongenesis [78]. *Ship1*-knockout mice can also develop myoproliferative syndromes, which suggests that PtdIns(3,4)P_2 is relevant in transmitting mitogenic signals [79].

4.3 Phospholipase C-γ

Phospholipase C-γ (PLC-γ) binds to a variety of RPTKs, including EGFR [80], Her2 [81], PDGFRα [82], TrkA [83] and TrkB [84], among others, where it is phosphorylated on several tyrosine residues [85]. PLC-γ catalyzes the

formation of inositol (1,4,5) triphosphaphate (IP_3) and diacylglycerol (DAG) from PtdIns(4,5)P_2. These two products, IP_3 and DAG, act as potent second messengers. IP_3 is a soluble molecule and will diffuse through the cytoplasm until binding to IP_3 receptors present on the surface of the endoplasmic reticulum. These receptors, ligand-gated Ca^{2+} channels, subsequently release Ca^{2+} into the cytoplasm. The protein kinase C (PKC) family protein kinases are then activated as a consequence of interaction with Ca^{2+}, which relieves intramolecular inhibitions. Furthermore, the binding of DAG localizes these proteins to the plasma membrane. There, PKC family enzymes phosphorylate a number of proteins involved in cellular division, leading to the activation of NFAT, NFκB and other proteins [86].

5. Termination of RPTK signaling

Not surprisingly, normal RPTK signaling involves numerous mechanisms that limit the magnitude and duration to which an activated receptor can propagate a signal. Receptor dephosphorylation and endocytosis are the most well described mechanisms and will be discussed in this section.

5.1 RPTK dephosphorylation

Members of the RPTK family are targeted by tyrosine phosphatases that, in general, act to limit the duration of signaling. However, numerous examples of tyrosine phosphatases whose action is of an activating nature exist, a classical example being the PTPα-mediated dephosphorylation of c-Src at

Tyr527 [87], which is required for c-Src activation [88]. Around 100 genes code for enzymes that are capable of cleaving phosphate from phosphotyrosine [89]. Members of the protein tyrosine phosphatase (PTP) family comprise the largest portion of these enzymes and are defined by an active site motif, HCX_5R, in which the cysteine residue acts as a nucleophile and is essential for catalysis [90]. PTP members include phosphotyrosine-specific (classical) phosphatases, which consist of both receptors (RPTPs) and soluble proteins. Other PTP members are dual-specificity phosphatases (DSPs) that recognize phosphotyrosine and/or phosphoserine/threonine and are strictly soluble proteins [91].

Some RPTPs have been shown to engage in ligand-dependent dimerization. In contrast with RPTKs however, these proteins appear to be de-activated by ligand binding, which may cause their active sites to become occluded [92]. The binding of pleiotrophin to RPTPζ is a well-characterized example of this phenomenon. Binding causes enhanced phosphorylation of β-catenin [93] and β-adducin [94], proteins that are involved in cadherin–catenin cell–adhesion complexes and actin filament regulation, respectively. These findings suggest that pleiotrophin-RPTPζ binding regulates the organization of the cytoskeletal architecture by diminishing RPTPζ activity. Although the ligands for many RPTPs have yet to be discovered, the presence of Ig-like and fibronectin type III domains extracellular domains in many of these proteins suggest a broad role in regulating cell-cell contact.

The activity of soluble PTPs is also tightly regulated, as evidenced by the variety of protein-protein interaction domains present in these proteins. For instance, SHP proteins are targeted to receptors and other scaffolding proteins via an SH2 domain [95]. Other soluble PTPs contain FERM, SEC14 and BRO1 domains that target the protein between the cytoskeleton and membrane [96], to lipids [97], and to endosomes [98], respectively. Furthermore, these PTPs contain intrinsic specificity towards their substrates. For instance, the tyrosine-phosphorylated activation loop of the insulin receptor is targeted directly by PTP1B, resulting in decreased protein-tyrosine kinase activity [99]. The solved structure PTP1B in complex with a peptide modeling the phosphorylated activation segment of the insulin receptor shows numerous, specific contacts; mutation of many of these residues results in diminished catalysis [99].

Notably, while members of the PTP family are all related, several non-PTP family members have also been recently discovered that can dephosphorylate tyrosine residues. Eya1 uses an aspartic acid-based motif to dephosphorylate Tyr142 of the histone H2AX [100]. Here, Eya1 activity promotes cell survival under a number of stresses [101]. STS-1 [102], also contains a protein-tyrosine phosphatase domain [103] related to the phosphoglycerate mutase (PGM) superfamily, with demonstrated activity on Syk [104] and other proteins [105]. Additionally, prostatic acid phosphatase (PAcP) was demonstrated to directly target ErbB-2 [106]. Interestingly, the level

of PAcP in cancer cells correlated with higher growth rates in xenograft animal models [107].

The DSPs show little sequence conservation outside of the signature HCX_5R active site motif and despite their name, individual DSPs often show preference for either tyrosine or serine/threonine. A sub-division of the DSPs, the MAPK phosphatases, exhibit diverse patterns of expression, localization and specificity for individual MAPKs. VHR for example, dephosphorylates a tyrosine residue in MAPKs [108]. Thus, many of these phosphatases act as critical regulators in attenuating RPTK-induced Ras-Erk activation.

5.2 Receptor endocytosis

Like many receptors, RPTK internalization is typically enhanced upon ligand binding. This a critical mechanism in the regulation of signaling from RPTKs. Interestingly, the reduction in the number of surface receptors, does not always diminish the maximum signaling response that can be generated. Instead, this process can shift the dose-response curve, such that a higher concentration of ligand is required to obtain a maximum signal. As mentioned, ligand binding followed by RPTK autophosphorylation induces the association of SH2- and PTB-domain containing proteins, some of which are directly involved in the transit of the receptor into endosomes. Receptors are then sorted into lysosomes for degradation or shuttled back to the cell surface and recycled.

RPTK internalization is dominated by clathrin-mediated endocytosis (CME). RPTKs utilize multiple mechanisms, such as ubiquitination, to enter CME.

Ubiquitination leading to CME involves E3 ubiquitin ligases that are recruited (either directly or through an adaptor) to the receptor (Figure 7). For instance, the adaptor Grb2 can recruit the E3 ubiquitin ligase c-Cbl to ubiquitinate the EGFRs and Met receptors [109,110]. Ubiquitinated receptors are then recruited to clathrin-coated pits (CCPs) via interaction with UBA domain present on proteins such as Epsin [111]. Epsin then binds the AP2 complex and clathrin to promote the formation of clathrin-coated pits. CCPs then undergo a “pinching off” and eventually form intracellular vesicles. After shedding many components of CCP, these vesicles fuse with early endosomes, which are defined by the presence of Rab5 [112]. The Rab-family proteins are small GTPases that recruit effectors involved in vesicle formation, transport and fusion [113]. Here, receptors can be recycled by a Rab4-dependent mechanism or join into multi-vesicular bodies. Ubiquitination, specifically the presence of lysine 48-linked polyubiquitination, then serves as recognition signal to target the protein for proteasomal degradation by a Rab7-dependent mechanism [114] (Figure 7). Notably, EGFR lacking the ubiquitin-conjugation sites display enhanced signaling [115], whereas other RPTKs display accelerated degradation and down-regulation in signaling in the absence of deubiquitylases (which are responsible for removing ubiquitin) [116]. Moreover, during fly oogenesis, it has been demonstrated that depletion of c-Cbl results in the

delocalization of RPTK signaling, which occurs at the leading edge of border cells during normal development [117]. These observations suggest that ubiquitination provides an important, if not critical, mechanism leading to the signaling, internalization and sorting of RPTKs.

The pH of endosomes also decreases as they mature and finally merge with lysosomes. Acidification is primarily mediated by the vacuolar ATPase, which pumps protons from the cytoplasm [118]. For some receptors, this promotes dissociation of ligand and recycling of the receptor back to the plasma membrane. For other receptor-ligand pairs, acidification does not cause dissociation. For instance, the EGFR remains bound to EGF within late endosomes.

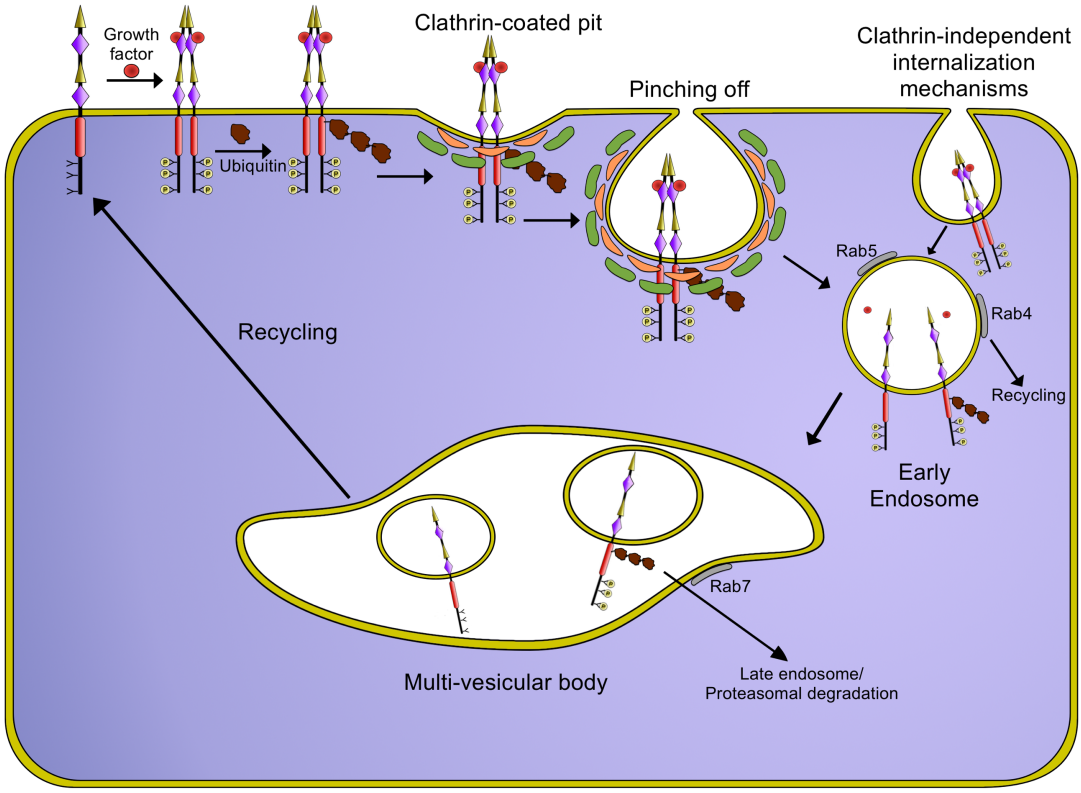

Figure 1.8 Receptor recycling and degradation
Following stimulation, RPTKs undergo internalization by clathrin and clathrin-independent mechanisms. Ubiquitination of receptors can accelerate their internalization due to the association of ubiquitin with components of clathrin-coated pits. Internalized vesicles arrive at Rab5-positive early endosomes where the ligands dissociate due to the acidic environment. Here, receptors can undergo recycling by a Rab4-dependent mechanism or are further processed into multi-vesicular bodies, where they no longer propagate signals. There, receptors can be recycled back to the plasma membrane or face degradation when marked for destruction. Here, a polyubiquitinated receptor is shown in transport to late endosomes by a Rab11A-dependent mechanism. Late endosomes then fuse with the proteasome, resulting in receptor degradation. The Rab-family proteins are small GTPases that recruit membrane effectors that are responsible for vesicle formation, movement, and fusion. Phosphatases, such as PTP1B, can dephosphorylate the receptors during their transit.

Importantly, most RPTKs appear to continue to signal after internalization. The EGFR, for instance remains ligand bound and phosphorylated until the late stages of endosomal trafficking [119]. The presence of all basic components of the Ras-Erk cascade (Grb2, Sos, Ras, Raf, Mek1/2, Erk1/2), suggests that critical signaling may emanate from endosome-

bound RPTKs. However, while some experimental systems show that RPTK internalization is required for maximal Erk activation [120,121], other systems suggest otherwise [122,123]. In all likelihood, the relative importance of this phenomenon varies by cell type, receptor-ligand identity, receptor-ligand concentration and other variables. Clearly though, the internalization of RPTKs plays a critical role in regulating expression levels as well as signaling specificity.

6. The impact of protein-tyrosine kinase research on cancer treatment

The development of imatinib to treat a variety of cancers is testimony of the ability of cell biologists, working with doctors, chemists and others to rationally design therapies that address the molecular root cause of human maladies. A (very) brief summary of the route to the development of imatinib is given below, followed by a discussion of tyrosine kinase signaling in regards to the treatment of cancer.

6.1 Development of imatinib and other RPTK inhibitors

The field of tyrosine kinase signaling was arguably kicked off around 1979 when research groups reported viral proteins (v-Abl [124], v-Src [125]) that catalyzed the phosphorylation of tyrosine residues. In 1980, the Cohen group discovered that A431 cells, when stimulated with EGF, can incorporate phosphate onto tyrosine residues of membrane-bound proteins [126]. It was well known at the time that EGF induced cell growth and division in some cell

types through complex changes in cell biology. Purified EGFR was also shown to engage in autophosphorylation on tyrosine residues, demonstrating that these receptors can behave as both enzyme and act as their own substrates. At the time, only serine and threonine were known to be phosphorylated in the context of normal cell physiology. In the following decade a dozen or so RPTKs were identified, many of which were demonstrated to engage in ligand-induced tyrosine phosphorylation [127].

Early insight into the function of these molecules came from the observation that the kinase domain of these newly discovered RPTKs showed remarkable similarity to kinase domain of v-Src, an oncogene derived from the Rous sarcoma virus (RSV). Because v-Src is essential for RSV to form tumors in chickens [128], it was hypothesized that tyrosine kinase activity regulates cell division and, when deregulated, cause tumors. Additionally, the finding that a *v-Src* homolog exists in animals [129], namely *c-Src,* suggested that human genes could be altered (mutated) such that they induce cancer; today we call these genes proto-oncogenes.

The BCR-Abl fusion protein, a hallmark of chronic myelogenous leukemia (CML), was soon discovered to be a potent tyrosine kinase [130]. BCR-Abl, a result of fusion of the *BCR* and *Abl* genes, results in a constitutively active tyrosine kinase due to the ability of the BCR domain to dimerize, allowing the Abl kinase domain to autophosphorylate and promote cell division in CML.

Subsequently, the BCR-Abl fusion was shown to induce CML-like disease in mice, proving that it was the primary causative agent of CML [131–134].

With the development of a potent BCR-Abl inhibitor, CGP571148B, which was capable of curing mice bearing BCR-Abl-induced tumors [135,136], clinical trials began in humans. These trials showed that CGP571148B, by then called STI571 and later imatinib, proved very effective at treating early-stage CML [137]. These results led to imatinib, which would be marketed under the name Gleevec in the U.S., to be approved to treat CML in 2001. In addition to CML and several other blood cancers, imatinib has been approved to treat gastrointestinal stromal tumors (GIST), in which an activated form of c-Kit is expressed.

From to the insight provided tyrosine kinase signaling research, several other rationally designed drugs to treat cancer resulting from aberrant protein-tyrosine kinase activity have been developed. For instance, trastuzumab (marketed as Herceptin) is a monoclonal antibody that is designed to target the RPTK HER2. HER2 is overexpression is strongly associated with more aggressive disease and higher recurrence rate in many breast cancers. Trastuzumab acts by binding to HER2 receptors and initiates antibody-dependent cell-mediated death of cancer cells, among other proposed mechanisms [138]. Currently, dozens of therapies consisting of monoclonal antibodies, small molecule inhibitors and immunotoxins are being developed to target specific, aberrantly signaling RPTKs.

6.2 Current limitations of RPTK-targeting agents

Having abandoned service to their host, cancer cells essentially exist in their own individual evolutionary arms races. Because of this, therapies that initially appear effective against a cancer can quickly lose their efficacy as individual cancer cells develop mutations that allow them to circumvent a particular focused therapy. Free from the growth constraints imposed by the therapy, these cells can continue to proliferate.

A classic example of this occurs with the use of “ATP-mimetic” tyrosine kinase inhibitors (TKIs) targeting the EGFR, which act by binding the active site of the kinase. The single EGFR mutation T790M has the dual effect of increasing receptor affinity for ATP and decreasing the affinity for TKIs. The net result is that TKIs become unable to outcompete ATP (which is at a relatively high intracellular concentration) for the receptor, thus rendering the therapy ineffective in cells with this mutation [139].

Ligand overexpression is another mechanism by which cells can overcome a targeted therapy. As mentioned, trastuzumab is an antibody targeting the extracellular portion of the EGFR. One route to resistance is achieved through enhanced autocrine EGF expression, which interferes with trastuzumab-binding to the EGFR [140]. In this situation, cancer cells secreting large amounts of EGF will be “protected”, rendering treatment with the antibody ineffective.

Activating mutations in proteins downstream of an RPTK play a role in the failure of some cancer treatments. For example, colorectal cancers are often responsive to the small-molecule inhibitors gefitinib and erlotinib, which target commonly mutated variants of the EGFR. However, resistance to these therapies often arises due to activating mutations in NRAS, KRAS and BRAF [141], which abolish therapeutic efficacy of these treatments. In fact, mutations and amplifications of KRAS are detected in >50% of patients being treat for colorectal cancer that has developed resistance to EGFR-targeting small-molecule inhibitors [142].

Multiple mutations almost always accompany the transformation of a healthy cell to one that can pathologically proliferate. Additionally, as cancer progresses and the genomic stability of the cell is compromised, mutations are acquired at rates much greater than the surrounding healthy tissues. Therefore, it is unlikely that a single therapy targeting oncogenic RPTKs will lead to sustained remission in a cancer patient. Going forward, continued development of more potent and selective inhibitors that are used in combination based on knowledge of the mutations present in the cancer should improve their therapeutic application. Targeting effectors, both upstream and downstream of RPTKs, will prevent the expansion of cells that have found mutations “around” a therapy. Moreover, identification of novel proteins that are critical “signaling hubs”, should foster the development of therapies that target critical components of the cell’s signaling machinery, thus decreasing the likelihood

that resistance to the therapy will arise due to the further development of activating mutations.

7. Protein-tyrosine kinase signaling research today

As cellular signaling pathways are elaborated, it becomes ever clearer that these pathways are better described as complex, interdependent webs that have numerous levels of regulation and redundancy. The growth of high throughput assays and the ability to perform comprehensive proteomic/genomic analysis has fostered a broader understanding of these systems. A selection of current interesting research in the field of tyrosine kinase signaling will follow.

7.1 Kazlauskas's two-pulse scenario updated

While continuous exposure to growth factors is sufficient for cells to progress through the cell cycle, it has also been demonstrated that two appropriately-timed pulses of growth factor are sufficient for cells to cross the restriction point of the cell cycle [143]. This finding has allowed scientists to analyze the specific events that occur following growth factor exposure to gain better insight into the minimum requirements for a cell to divide. Recently, the Yarden group studied this phenomenon by performing comprehensive proteomic and transcription analysis of normal mammary epithelial cells following pulsed exposure to growth factors in order to identify the mechanisms that allow these cells to divide under the two-pulse protocol [144]. Their results show that the initial pulse of EGF initiates enhanced protein expression,

including genes associated with lipid biosynthesis, in addition to modest activation of Erk. Additionally, this initial pulse activated antiproliferative signaling involving p53 activation. In the absence of an EGF pulse exactly 7 hours later, the activation of p53-mediated anti-proliferative genes proved to be sufficient to prevent entry into S-phase, by preventing restriction point crossing, thus preventing cell division. Cells pulsed with EGF for a second time at 7 hours display PI3K/Akt activation that is required to suppress the anti-proliferative genes activated by the first pulse. Additionally, these cells display enhanced Erk activation (as compared to the first EGF pulse), leading to enhanced EGR1 expression, both of which were necessary for the cells to overcome the restriction point.

Besides revealing insight into the specific mechanism of tyrosine kinase signaling, the results of this study also provides several important examples of regulation in regards to tyrosine kinase signaling. Firstly, RPTKs can activate a multitude of signaling pathways that are dependent on cellular context, in this case the state of the cell cycle. In the first EGF pulse, modest Erk activation and p53 activation was observed. The second EGF pulse, however, activates PI3K/AKT and Erk, while downregulating the expression of anti-proliferative genes that were activated by the first EGF pulse. In this system, PI3K/Akt activation specifically interferes with the activity of p53; this provides an example “cross-talk” that is a common feature of cellular signaling. Furthermore, a threshold mechanism ensures that the cells divide only when a strong-enough

signal is received as a sufficient amount of activated Erk and EGR1 is absolutely required for these cells to replicate.

7.2 van der Geer laboratory

In our lab, we study the role of ShcA in tyrosine kinase signaling. As mentioned above, ShcA is an adaptor protein that is recruited to activated RPTKs. ShcA binds these receptors in the context of phosphotyrosine where it too is phosphorylated on several tyrosine residues. These phosphotyrosine residues then act as binding sites for other proteins, such as Grb2 [145]. Interestingly, some RPTKs bind Grb2 directly, whereas as others bind ShcA directly and thereby recruit Grb2. We recently began to investigate the hypothesis that ShcA's role in this context is more than to recruit Grb2. To evaluate this, we engineered nerve growth factor receptors to bind ShcA, Grb2, or neither (null) and assayed their ability to activate signaling [146]. We found that cells expressing Grb2-binding receptors behaved similarly to cells expressing ShcA-binding receptors in terms of Ras-Erk and Akt activation. Interestingly though, cells expressing the ShcA-binding receptors display accelerated cell division as compared to cell expressing the Grb2-binding receptors. One explanation of this result is that ShcA recruits additional proteins into the signaling complex that are not present when receptors bind Grb2 directly. In the remainder of this manuscript, I will describe our efforts to identify

novel ShcA-binding proteins and characterize their role in signal transduction in regards to RPTKs and ShcA.

Chapter 2. Identification of STS-1 as a novel ShcA-binding protein

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Identification of STS-1 as a novel ShcA-binding protein

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ABSTRACT

ShcA is a cytoplasmic signaling protein that supports signal transduction by receptor protein-tyrosine kinases by providing auxiliary tyrosine phosphorylation sites that engage additional signaling proteins. The principal binding partner for tyrosine phosphorylation sites on ShcA is Grb2. In the current study, we have used phosphotyrosine-containing peptides to isolate and identify STS-1 as a novel ShcA-binding protein. Our results further show that the interaction between STS-1 and ShcA is regulated in response to EGF receptor activation.

1. Introduction

Receptor protein-tyrosine kinases mediate the regulation of various cellular processes, including growth, cell division, differentiation, and migration, in response to extracellular signals [1]. Ligand binding causes receptor oligomerization, activation of the protein-kinase domain, and autophosphorylation on one or more tyrosine residues in the cytoplasmic region of the receptor [2]. Receptor autophosphorylation sites generally serve as binding sites for cytoplasmic signaling proteins [3,4]. The binding of signaling proteins to receptor autophosphorylation sites is mediated by SH2 or PTB domains [5]. These domains bind to phosphorylated tyrosine residues that are present within specific sequence motifs [6]. As a consequence of their interaction with the receptor, signaling proteins propagate signals from the receptor into the interior of the cell, ultimately leading to changes in cellular physiology [3,4,7].

The ShcA gene encodes three proteins that differ from each other exclusively in the length of an amino-terminal region [8]. This region is followed by a PTB domain, a central region containing several tyrosine phosphorylation sites, and a carboxy-terminal SH2 domain [9]. ShcA proteins bind to specific tyrosine phosphorylation sites on cell surface receptors [10]. Following binding to a receptor, ShcA is phosphorylated on tyrosines 239, 240, and 317, thereby providing the receptor with additional binding-sites for SH2 domain-containing signaling proteins [11,12].

The principle binding partner for tyrosine phosphorylation sites on ShcA is Grb2 [13]. Grb2 is a small adaptor protein that uses its SH2 domain to bind to ShcA and its SH3 domains to bind to Sos and Gab [14–16]. Sos is an upstream activator of Ras [17]. Gab proteins contain phosphotyrosine-based docking sites for various signaling proteins, including PI 3-kinase [18]. Thus, ShcA and Grb2 connect receptors with the Ras-MAP kinase and PI 3-kinase-Akt pathways [18,19]. While some receptors depend on ShcA for the recruitment of Grb2, others can bind Grb2 directly, and there are even some receptors that will bind directly to both ShcA and Grb2 [20]. This observation led us to question the commonly held belief that ShcA functions exclusively to provide binding sites for Grb2 [20].

To identify novel proteins that bind to ShcA in a phosphotyrosine-dependent manner, we used synthetic peptides identical to ShcA phosphorylation sites as affinity reagents. This approach led to the identification of STS-1 as a novel ShcA-binding partner.

2. Materials and methods

2.1. Reagents

EGF and the anti-phosphotyrosine monoclonal antibody 4G10 were purchased from Fisher Scientific (Waltham, MA). A polyclonal anti-STS-1 serum was purchased from Abcam (Cambridge, MA). A polyclonal anti-serum was raised against a GST-ShcA-SH2 domain fusion protein [21]. The polyclonal anti-Grb2 serum was a gift from Dr. T. Hunter [22]. Protein A-horse radish peroxidase (HRP), sheep anti-mouse-HRP and donkey anti-rabbit-HRP were purchased from Sigma-Aldrich (St. Louis, MO). Protein A-agarose was purchased

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from Repligen (Waltham, MA).

2.2. Cell lines, stimulation, and lysis

A549 cells and COS1 cells were grown on 10 cm tissue culture plates in DMEM supplemented with 10% fetal bovine serum. Prior to stimulation, the cells were starved for 12–16 h in DMEM with 20 mM HEPES, pH 7.2. Control cells and cells stimulated with 50 ng/mL EGF were lysed in PLC-lysis buffer as described [21].

2.3. Peptide affinity purification and silver staining

Unphosphorylated and phosphorylated peptides based on the ShcA Tyr 239/Tyr 240 and Tyr 317 phosphorylation sites were synthesized by AnaSpec (Freemont, CA). All peptides were synthesized with an amino-caproic acid linker at the amino-terminus. Peptides were linked through the amino-terminal amino group to AminoLink agarose at 1 mg/mL in pH 10.0 coupling buffer, according to the manufacturers instructions (Thermo Fisher Scientific, Waltham, MA).

Clarified cell lysates were incubated with 20 μL 50% peptide-agarose slurry per mL of lysate on a rocker for 1 h at 4°C. Beads were washed four times with 1 mL PLC-lysis buffer. Bound proteins were resolved by SDS-PAGE and visualized by silverstaining as described [21,23].

2.4. Mass spectrometry

To obtain enough protein to identify p72 by mass spectrometry, A549 whole cell lysate from ten 10 cm dishes was incubated with 40 μL of a 50% slurry of p.Y317 peptide-agarose. Following SDS-PAGE, proteins were visualized using SimplyBlue SafeStain, according to the manufacturer's directions (Thermo Fisher Scientific, Waltham, MA). The protein band of interest was excised from the stained gel, de-stained with 40% n-propanol and 50% acetonitrile, and digested over 16 h at 37 °C with 100 ng trypsin in 10 μL 20 mM ammonium bicarbonate. Peptides were extracted, dried completely, and dissolved in 0.1% formic acid for ESI-MS/MS analysis on a Thermo LTQ-Orbitrap instrument. Peptide precursor masses were determined with high accuracy by Fourier-transform MS in the Orbitrap followed by data dependent MS/MS of the top 5 precursor ions in each chromatographic time window. Data were analyzed using the Mascot algorithm (Matrix Science, London, UK) on a local Mascot server (version 2.1.0) and searched against the latest NCBI non-redundant protein database.

2.5. Immunoprecipitation and immunoblotting

Immunoprecipitations, obtained by incubation of cell lysates with 1–5 μL of polyclonal antiserum and 100 μL 10% protein A-agarose, were resolved by SDS-PAGE and visualized by immunoblotting as described [21].

3. Results

3.1. Multiple proteins bind in a phosphorylation-dependent manner to ShcA-derived peptides

ShcA signaling proteins are composed of an amino-terminal PTB domain, a central region containing several tyrosine phosphorylation sites, and a carboxy-terminal SH2 domain (Fig. 1A). It is widely accepted that both the Tyr 239/Tyr 240 and the Tyr 317 phosphorylation sites act as binding-sites for Grb2. To investigate whether other proteins bind to these sites as well, we used ShcA-based phosphopeptides as affinity reagents (Fig. 1B). Unphosphorylated and phosphorylated synthetic peptides identical to ShcA tyrosine phosphorylation sites, were covalently linked to agarose beads and incubated with lysates of African green monkey COS-1 cells. Bound proteins were resolved by SDS-PAGE and visualized by silver staining (Fig. 1C). Several proteins, with masses between 43 and 70 kDa, bound nonspecifically to all five peptides. A protein with a mass of approximately 20 kDa bound to both the doubly phosphorylated Tyr 239/Tyr 240 peptide and to the phosphorylated Tyr 317 peptide (Fig. 1C, lanes 3 and 5). In addition, we observed specific binding to the p.Tyr 317 peptide of a 72 kDa protein and a 120 kDa protein (Fig. 1C, lane 5). To confirm these results in a different species, the ShcA peptide beads were incubated with lysates of A549 human adenocarcinoma cells. Bound proteins were resolved by SDS-PAGE and visualized by silver staining (Fig. 1D). The results were similar to those observed with the COS-1 cells. A 20 kDa protein bound specifically to both the p.Tyr 239/p.Tyr 240 and the p.Tyr 317 peptides (Fig. 1D, lanes 3 and 5). We also found specific binding of the 72 kDa protein to the p.Tyr 317 peptide (Fig. 1D, lane 5). In addition, several minor binding partners for the p.Tyr 317 peptide were present in the human A549 cells (Fig. 1D, lane 5). Thus, ShcA-based phosphopeptides bind to several different proteins in lysates of mammalian tissue culture cells.

3.2. Identification of p72 as STS-1

To identify the 72 kDa protein that binds specifically to the p.Tyr 317 peptide, the affinity purification described above was scaled up and carried out with 5-10 $\times$ 10^7 A549 human adenocarcinoma cells. Following SDS-PAGE and colloidal Coomassie blue staining, the 72 kDa protein was excised from the gel, digested with trypsin, and analyzed by tandem mass spectrometry. Our experiment yielded 16 peptide sequences that matched suppressor of T-cell signaling 1 or STS-1 (Fig. 2A). These peptides represented 21% of the amino acid sequence of STS-1 and were distributed evenly along the polypeptide chain, thereby showing good coverage of the protein.

To confirm the identification of STS-1 as a ShcA-binding protein, cell lysates from starved COS-1 cells were incubated with the immobilized ShcA peptides. Bound proteins were analyzed by anti-STS-1 immunoblotting. Whole cell lysates from control and EGF-stimulated COS-1 cells were included as controls. The results show that the anti-STS-1 antiserum detects a single protein band in COS-1 cell lysates (Fig. 2B, lanes 1 and 2). It appears that the amount of STS-1 in whole cell lysates decreases in response to stimulation with EGF (Fig. 2B, compare lanes 1 and 2). Of the five peptides tested, only the p.Tyr 317 peptide bound to STS-1 (Fig. 2B, lane 7).

To verify that the ~20 kDa protein that was observed to bind to two of the ShcA phosphopeptides indeed represents Grb2 (Fig. 1C and D, lanes 3 and 5), peptide bound proteins were also analyzed by anti-Grb2 immunoblotting. The results confirm that this protein represents Grb2 and that Grb2 binds to both the p.Tyr 239/p.Tyr 240 peptide and the p.Tyr 317 peptide (Fig. 2C).

Thus, peptide affinity purification can be used to isolate ShcA-binding proteins. Using peptide affinity purification and mass spectrometry, we identified STS-1 as a novel ShcA-binding partner. The identification was confirmed by anti-STS-1 immunoblotting.

3.3. The association of STS-1 and ShcA is responsive to stimulation with EGF

To further investigate the interaction between ShcA and STS-1 in the context of EGF receptor activation, COS-1 cells were stimulated with EGF for varying amounts of time. ShcA proteins were subsequently isolated by immunoprecipitation and analyzed by anti-STS-1 immunoblotting (Fig. 3A). Anti-p.Tyr and anti-ShcA immunoblots

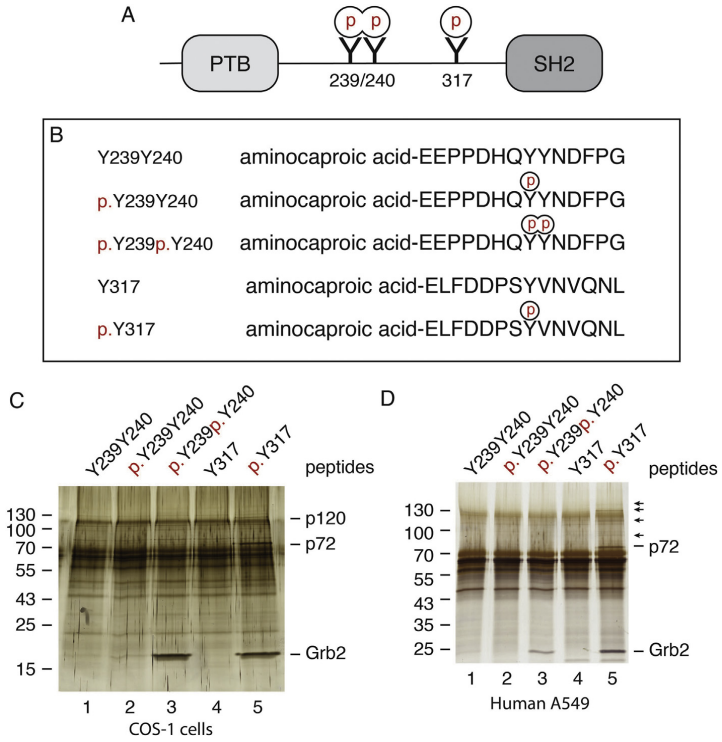

Fig. 1. Multiple proteins bind the ShcA-derived peptides in a phosphorylation-dependent manner. (A) ShcA contains a PTB domain that binds Asn-Pro-X-p.Tyr motifs, a central region containing tyrosine phosphorylation sites, and a carboxy-terminal SH2 domain that binds to tyrosine phosphorylation sites [24]. (B) Five peptides based on the sequence surrounding the Tyr 239/Tyr240 and Tyr 317 phosphorylation sites were synthesized and coupled to agarose beads. An aminocaproic acid residue at the amino-terminus was used a spacer. COS-1 (C) and human A549 (D) cell lysates were incubated with unphosphorylated and phosphorylated ShcA peptides linked to agarose beads. Bound proteins were resolved by SDS-PAGE and visualized by silverstaining.

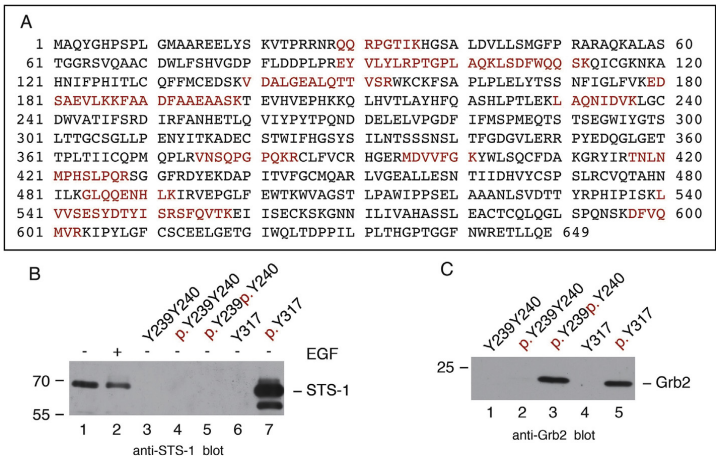

Fig. 2. Identification of STS-1 as a ShcA-binding protein. (A) The 72 kD protein purified by peptide affinity chromatography from A549 human carcinoma cells was resolved from contaminants by SDS-PAGE, excised from the gel, digested with trypsin, and analyzed by two-dimensional mass spectrometry. Sixteen peptide sequences resulting from this analysis matched the STS-1 sequence and are shown in red. (B) Whole cell lysates from starved COS-1 cells were incubated with immobilized ShcA peptides (lanes 3–7). Bound proteins were analyzed by anti-STS-1 immunoblotting. Whole cell lysates from control and EGF-stimulated cells were analyzed in parallel (lanes 1 and 2). (C) Whole cell lysates from starved COS-1 cells were incubated with immobilized ShcA peptides and bound proteins were analyzed anti-Grb2 immunoblotting. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were included as controls (Fig. 3, panels B and C). The experiment shows association between ShcA and STS-1 in unstimulated cells (Fig. 3A, lane 1). This association is consistent with the presence of tyrosine phosphorylated ShcA in unstimulated cells (Fig. 3B, lane 8). The amount of ShcA-bound STS-1 went up during the first 2 min of stimulation (Fig. 3A, lanes 2 and 3), after which association declined (Fig. 3A, lanes 4–7). The onset of the decline does not appear to correlate with a reduction in ShcA tyrosine phosphorylation or ShcA protein levels (Fig. 3, panels B and C).

To visualize STS-1 protein levels, STS-1 immunoprecipitates were analyzed by anti-STS-1 immunoblotting. The results show that STS-1 levels start to decline within 1 min after addition of EGF (Fig. 3D). Therefore, the transient increase in binding of STS-1 to ShcA is a result of the increase in ShcA tyrosine phosphorylation and the decrease in STS-1 protein levels that occur in response to EGF. In summary, these results demonstrate that the STS-1 protein levels decrease, and its association with ShcA transiently increases during activation of the EGF receptor protein-tyrosine kinase.

4. Discussion

ShcA is a docking protein that is recruited to active growth factor receptor-protein tyrosine kinases [24]. It is equipped with a PTB domain, several tyrosine phosphorylation sites, and an SH2 domain to bring together proteins in a signaling complex. A recent study in which ShcA-containing signaling complexes were isolated and analyzed showed that ShcA-dependent protein-protein interactions change with the time after stimulation [25]. Proteins that associate early after stimulation appear to be involved in transmitting the stimulatory signal, while proteins that associate at later times appear to be involved in termination of signaling [25]. This study also concluded that the interactions that are dependent on ShcA tyrosine phosphorylation are largely dependent on Grb2 [25].

It is well established that tyrosine phosphorylation site-binding domains bind to phosphorylated tyrosine residues in the context of their primary sequence [26,27]. Based on this principle, we previously pioneered the use of phosphopeptides as affinity reagents for the purification of tyrosine phosphorylation site-binding proteins [28]. In the current study, we have used this approach to purify ShcA-binding proteins. As expected, we observed binding of Grb2 to the p.Tyr 317 peptide (Figs. 1 and 2). Tyr 317 has been characterized extensively as a Grb2-binding site [29–31]. In addition, our results show that Grb2 also binds to the doubly phosphorylated p.Tyr 239/p.Tyr 240 peptide (Figs. 1 and 2). This is in agreement with previous studies on ShcA phosphorylation site mutants [11,12]. Interestingly, the singly phosphorylated p.Tyr 239/Tyr 240 peptide did not bind Grb2 (Figs. 1 and 2). This is unexpected because this peptide contains the p.Tyr-X-Asn sequence that has been identified as the consensus binding motif for the Grb2 SH2 domain [27]. It currently remains unclear how the two adjacent phosphorylated tyrosine residues present in the p.Tyr 239/p.Tyr 240 peptide are accommodated by the Grb2 SH2 domain. Furthermore, it remains to be established why the absence of a phosphate group on the second tyrosine in this peptide prevents or strongly reduces binding.

The p.Tyr 317 peptide was observed to bind to several different, unidentified proteins, the most prominent of which had an apparent molecular weight of 72 kDa (Fig. 1, panels C and D). This protein was identified by mass spectrometry as STS-1. STS-1 is a cytoplasmic signaling protein composed of 649 amino acids [32]. It contains an amino-terminal ubiquitin-binding domain, a region with homology to a family of histidine phosphoesterases, an SH3 domain, and a domain with homology to phosphoglycerate mutase [33–35]. Ubiquitin-binding domains bind to ubiquitin, SH3 domains are known to bind to polyproline helices, and the phosphoglycerate mutase domain has been shown to act as a protein-tyrosine phosphatase [34,36,37]. No function has been established for the STS-1 phosphoesterase domain.

STS-1 has previously been purified using peptide affinity chromatography from DA-3 mouse lymphoma cells [32]. In their experiment, Carpino and coworkers used a phosphorylated peptide based on the sequence around Tyr 966 in JAK-2. The phosphorylated peptide bound to several different proteins, including PLCγ, PI 3-kinase, ShcA, and STS-1 [32]. In their experiment Carpino and coworkers used 10^{11} cells in order to isolate enough STS-1 required for identification. The results presented here suggest the possibility that the phosphorylated JAK-2 peptide represents a binding site for ShcA and that STS-1 was associated with a fraction of the ShcA isolated in their experiment.

Dikic and co-workers identified STS-1 as a Cbl-binding protein [38]. Their experiments show that the STS-1 SH3 domain can bind to proline-rich sequences in Cbl. In their system, STS-1 appears to inhibit Cbl-dependent receptor downregulation. Our experiments show that STS-1 binds to tyrosine phosphorylated ShcA, which is know to bind to the EGF receptor as well, thus providing an alternative route to its interaction with the activated receptor [8].

STS-1 was not identified as a ShcA-binding protein in a recent study by Pawson and coworkers [25]. However, we noticed several

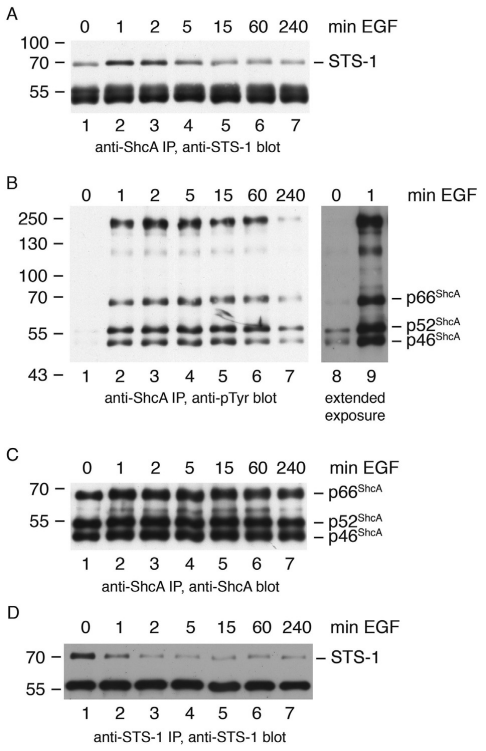

Fig. 3. Stimulation with EGF results in a transient increase in the association between STS-1 and ShcA. ShcA and STS-1 were isolated by immunoprecipitation from COS-1 cells that were stimulated with EGF for varying amounts of time. ShcA immunoprecipitates were analyzed by anti-STS-1 (panel A), anti-p.Tyr (panel B), and anti-ShcA (panel C) immunoblotting. STS-1 immunoprecipitates were analyzed by anti-STS-1 immunoblotting 1 (panel D).

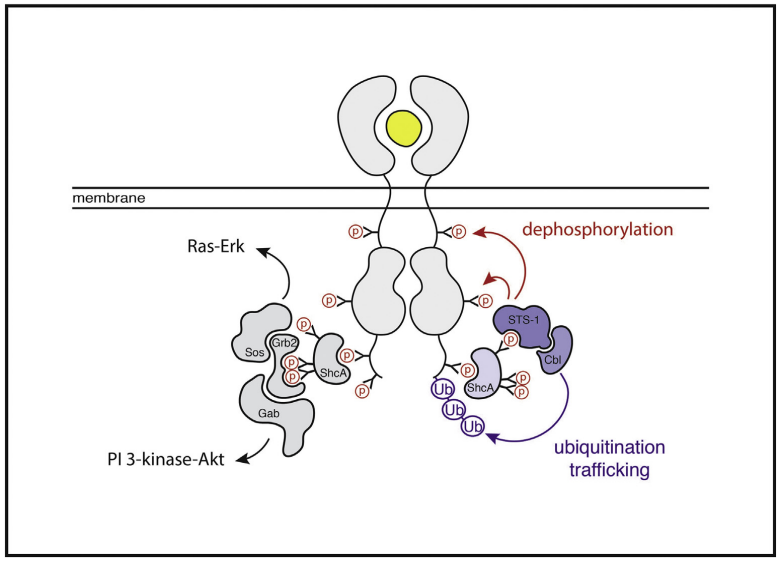

Fig. 4. An expanded role for ShcA in signal transduction. Receptor-protein tyrosine kinases undergo autophosphorylation on tyrosine residues in response ligand binding. Upon binding to receptor autophosphorylation sites, ShcA becomes phosphorylated on tyrosines 239, 240 and 317 [11,12]. It has been established firmly that ShcA plays a role in linking activated receptors to activation of the Ras-Erk and PI 3-kinase-Akt pathways [18,19]. In the current study we have identified STS-1 as a novel ShcA-binding protein that can bind specifically to the Tyr 317 phosphorylation site. STS-1 contains a phosphoglycerate mutase/protein phosphatase domain that could mediate dephosphorylation of ShcA or the receptor. In addition, STS-1 contains an SH3 domain has been shown to bind to Cbl [38]. Cbl and STS-1 could collaborate in the ubiquitination of the receptor, thereby regulating its trafficking or degradation.

differences between their investigation and ours. We identified STS-1 in A549, a human alveolar carcinoma cell line [39]. Pawson and coworkers used a rat fibroblast cell line [25]. While surveying cells, we observed dramatic differences in STS-1 protein levels between cell lines. Specifically, we observed very low levels of STS-1 in NIH3T3 mouse fibroblasts (results not shown). Thus, it is not unlikely that STS-1 was not detected in the Pawson study because it is expressed at low levels in fibroblasts. Additionally, Pawson and coworkers expressed as bait in their studies a version of the 52 kDa form of ShcA that was modified at its amino-terminus by addition of a FLAG epitope tag followed by the fluorescent green protein [25]. It is possible that these modifications affected ShcA localization in the cell or its ability to interact with some of its binding partners.

A time course experiment following stimulation of cells with EGF shows that the interaction between STS-1 and ShcA is responsive to activation of the EGF receptor. STS-1 binds to ShcA in unstimulated cells and binding goes up during the first 2 min after stimulation. Binding of STS-1 to ShcA in unstimulated cells is consistent with the presence of residual ShcA tyrosine phosphorylation (Fig. 3B, lane 8). The increase in ShcA tyrosine phosphorylation provides additional STS-1 binding sites, resulting in an increase in binding immediately after addition of EGF (Fig. 3A). However, within minutes after the onset of stimulation binding of STS-1 to ShcA declines. STS-1 immunoprecipitation experiments show that STS-1 protein levels decline rapidly after stimulation with EGF (Fig. 3D). Consequently, the transient increase in STS-1 association with ShcA results from the increase in binding sites on ShcA and a decrease in STS-1 protein levels that both occur in response to stimulation with EGF. Interestingly, Pawson and coworkers did identify a protein named Shcbp1 that was observed to have ShcA-binding characteristics that are similar to what we observed with STS-1 [25]. This may suggest that STS-1 and Shcbp1 represent a previously overlooked family of signaling proteins.

It is widely accepted that ShcA is recruited to receptors to provide a binding-site for Grb2. Grb2 uses its SH3 domains to bind Sos and Gab (Fig. 4). Sos is an upstream activator of Ras and Gab provides phosphotyrosine-based docking sites for several different proteins, including PI 3-kinase. Thus, the function of ShcA is to connect receptors to the Ras-Erk and PI 3-kinase-Akt pathways. Our results show that ShcA can also mediate the interaction of receptors with STS-1. STS-1 contains a phosphoglycerate mutase domain that has been shown to act as a protein-tyrosine phosphatase [34]. Thus STS-1 may act as a negative regulator of tyrosine phosphorylation (Fig. 4). Dikic and coworkers have shown that STS-1 can interact with Cbl [38]. Cbl is a ring-finger containing ubiquitin ligase [40]. Thus, ShcA may be involved in the regulation of receptor trafficking and degradation through its interactions with STS-1 and Cbl (Fig. 4). In this context, it is intriguing to note that NPXY motifs that are now known to act as ShcA PTB domain-binding sites were first discovered in the LDL receptor as internalization motifs [41].

Conflict of interest

All authors declare there is no conflict of interest.

Acknowledgements

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Chapter 2, in full, is a reprint of the material as it appears in Biochemical and Biophysical Research Communications, 2017. Identification of STS-1 as a novel ShcA-binding protein. van der Meulen, Talitha; Swarts, Spencer; Fischer, Wolfgang; van der Geer, Peter. The dissertation author is co-author and investigator of this material.

Chapter 3. Identification of the phosphotyrosine- and ShcA-binding domain in STS-1

Abstract

ShcA is an adaptor protein that is recruited to active receptor protein-tyrosine kinases where it is subjected to phosphorylation on several tyrosine residues, which then act as binding sites for Grb2. Many receptors also bind Grb2 directly. Differences in signaling from receptors that bind ShcA directly, compared to receptors that bind Grb2 directly suggest ShcA may recruit additional proteins into signaling complexes. Here, we demonstrate that STS-1 binds directly to a phosphopeptide representing the Tyr317 region of ShcA. Additional limited proteolysis and mutational analysis studies of STS-1 reveal that a novel phosphotyrosine-binding domain related to the 2H phosphoesterase superfamily mediates the interaction between STS-1 and ShcA. We also identify key residues in ShcA that are required for the interaction.

1. Introduction

The activation of receptor protein-tyrosine kinases (RPTKs) is associated with many cellular activities, including cell division, differentiation, growth and migration [147]. ShcA is an adaptor protein that is directly recruited to active (and thus tyrosine-phosphorylated) receptor protein-tyrosine kinases where it is subjected to phosphorylation on tyrosine residues 239, 240 and 317 [148]. The phosphotyrosine residues in ShcA then act as binding sites for yet other

proteins, such as Grb2 [145], and are critical components in linking receptor activation into biochemical changes within the cell, such as activation of signaling pathways including PI3K-Akt [149] and Ras-ERK [150].

We recently described a system in which cells expressing nerve growth factor receptors (NGFRs) that were engineered to bind Grb2 responded differently than cells expressing a form of the NGFR which binds ShcA directly [146]. Specifically, cells expressing the ShcA-binding NGFR showed higher levels of DNA synthesis following NGF stimulation than cells expressing the Grb2-binding NGFR [146]. These results led us to attempt to identify additional proteins that may be recruited into signaling complexes via interaction with ShcA tyrosine phosphorylation sites. We hypothesized that these ShcA-interacting proteins may explain the differences in signaling outcomes we observed.

Using peptides representing the phosphorylation sites in ShcA as reagents in affinity purification experiments, we recently demonstrated that STS-1 associates with a peptide representing the phosphorylated tyrosine-317 region (pTyr317 peptide) of ShcA, whereas STS-1 does not associate with the unphosphorylated form of the peptide (Y317 peptide) [151]. Furthermore, the association between full-length, endogenously expressed STS-1 and ShcA proved to be enhanced following growth factor stimulation [151].

These results led us to determine whether STS-1 and ShcA interact directly and, if so, which region of STS-1 mediates the interaction with

phosphorylated ShcA. Here, we demonstrate that STS-1 binds directly to the pTyr317 peptide using a domain with homology to the 2H phosphoesterase (2HPE) superfamily. Furthermore, using Ala-substituted pY317 peptides, we identify a critical residue in the pTyr317 peptide that is required for the interaction.

2. Materials and methods

2.1 Cells, STS-1 constructs, transfection and lysis

All cells were grown in DMEM supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, MA). Recombinant GST-STS-1 fusion proteins were generated by cloning cDNA for STS-1 into the pGEX-4T2 plasmid and purified as described elsewhere [60]. FLAG-STS-1 constructs with domain deletions or point mutations intended to inactivate each domain of STS-1 were designed using bioinformatic analysis and generated by PCR mutagenesis. The composition of domain-defective STS-1 constructs are as follows: ΔUBA, FLAG-STS-1(66-649); 2HPE8, FLAG-STS-1(His126Ala); 2HPE10, FLAG-STS-1(His212Ala); $SH3^-$, FLAG-STS-1(Trp295Leu); and PGM^-, FLAG-STS-1(Arg290Ala, His391Ala) (See figure 7 for relative locations of deletion/mutations). All constructs were sequenced to ensure presence of the desired mutation. Wild type and mutant constructs for FLAG-STS-1 were cloned into pcDNA3 vectors. For expression of these construct in cell-based assays, 10^6 HEK293 cells were seeded on 10 cm tissue culture dishes in DMEM with

10% FBS. 24 hours later, cells were transfected with 3 µg DNA using Lipofectin (ThermoFisher) according to manufacturer's recommendations. After a six-hour incubation, cells were placed on DMEM with 10% FBS. 48 hours later for transfected cells and in general for all other cell-based experiments, cells were placed in media containing DMEM with 20 mM HEPES pH 7.2. for 6 hours prior to lysis. Lysis proceeded by first rinsing cells twice with ice-cold 10 mM Tris-Cl pH 7.2, 150 mM NaCl (TBS), followed by lysis in 1 mL 50 mM HEPES pH 7.5, 150 mM sodium chloride, 10% glycerol, 1% Triton X-100, 1.5 mM magnesium chloride, 1 mM EGTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 100 µM sodium vanadate, 1 mM DTT, 1 mM AEBSF, 1.3 mM benzamidine, 1 µM E64, 1 µM leupeptin (PLC-lyis buffer). Cell lysates were collected in 1 mL microcentrifuge tubes, incubated on ice for 20 min, and clarified by centrifugation in a microcentrifuge at 10,000 rpm for 20 minutes at 4°C.

2.2 Immunoprecipitation and peptide affinity purification

Anti-FLAG immunoprecipitates were obtained by incubating cell lysates with 20 µL anti-FLAG M2 affinity gel (Sigma-Aldrich, St. Louis, MO) on a rocker for 1 hour at 4°C. For peptide affinity purifications, peptides based on the ShcA phosphorylation sites were synthesized by AnaSpec (Freemont, CA) as described elsewhere [151]. To perform peptide affinity purification, cell lysates or purified GST-STS-1 was incubated with 20 µL 50% peptide-agarose slurry on a rocker for 1 hour at 4°C.

2.4 Limited proteolysis of STS-1 and peptide sequencing

To identify the smallest fragment of STS-1 capable of binding to the pTyr317 peptide, limited proteolysis experiments were performed. 75 µL of purified GST-STS-1 (1mg/mL) was incubated with 1 µL of a 1:10 dilution of thermolysin (ThermoFisher) for three hours at room temperature and subjected to affinity purification with the pTyr317 peptide as described above. A fragment, p38, was excised from the gel and sent to the UC Davis Proteomics Core Facility for amino-terminal sequencing.

2.5 Coomassie staining and immunoblotting

Samples obtained by immunoprecipitation or peptide affinity purification, in addition to cell lysates, were denatured by boiling for 5 minutes in 62.5 mM Tris/Cl pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, and 0.025% bromophenol blue (SDS-PAGE sample buffer) and resolved by SDS-PAGE. For Coomassie staining, gels were incubated overnight with stain, followed by overnight destaining. For immunoblotting, samples were transferred to PVDF membrane. PVDF membranes were blocked for one hour in 10mM Tris/Cl pH 7.4, 150mM NaCl, 0.2% Tween 20 containing 5% milk (blocking solution) and then incubated for two hours with 2 µL of anti-FLAG antibodies (Sigma-Aldrich) or 2 µL anti-STS-1 antiserum in blocking solution. Membranes were then washed twice in 10mM Tris/Cl pH 7.4, 150mM NaCl, 0.2% Tween 20 (TBST) for 10 minutes, followed by two washes in 10mM Tris/Cl pH 7.4, 150mM NaCl

(TBS) for 5 minutes. Membranes were then incubated with a 1:10,000 dilution of goat anti-mouse-HRP for anti-FLAG antibodies or goat anti-rat-HRP for anti-STS-1 antibodies in TBST for 30 minutes and washed as described above and visualized by enhanced chemiluminescence [152].

3. Results

3.1 Mammalian cell lines express variable amounts of STS-1

To determine the expression profile of STS-1, several mammalian cell lines were surveyed by anti-STS-1 immunoblotting. Samples were obtained by subjecting cell lysates to affinity purification with the Tyr317 and pTyr317 peptides. We examined cells from African green monkeys (COS1), rats (C6, PC12), mice (NIH3T3) and humans (A549, 293A). STS-1 orthologs expressed in these cells share ~95% amino acid identity overall (data not shown). Similarly, the region of ShcA corresponding to the Tyr317 peptide is nearly identical in all species (data not shown). These findings suggest the interaction between STS-1 and ShcA is likely a conserved feature of mammalian cell signaling.

We found that the pTyr317 peptide purified STS-1 from COS1, A549, 293A and C6 cells (Figure 1, upper blot, lanes 2, 4, 6, and 10). This contrasts with the association of Grb2 with the pTyr317 peptide, which was detected in all cell lines examined (Figure 1, lower blot, even numbered lanes).

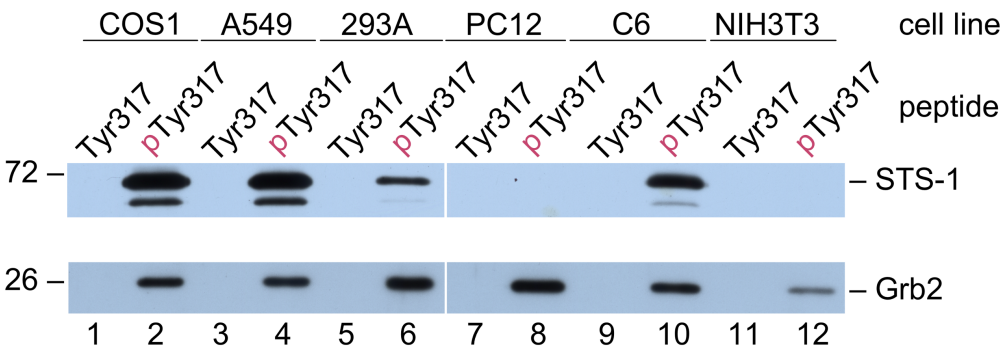

Figure 3.1 Variable amounts of STS-1 are purified from a variety mammalian cell lines
Lysates from cell lines derived from monkeys (COS1), rats (C6, PC12), mice (NIH3T3) and humans (A549, 293A) were subjected to affinity purification with the Tyr317 and pTyr317 peptides. These samples were analyzed by anti-STS-1 (upper blot) or anti-Grb2 (lower blot) immunoblotting. Upper blot, robust association of the pTy317 peptide with STS-1 was detected in COS1, A549, and C6 cells (lanes 2, 4, 10) whereas in 293A cells less STS-1 was detected (lane 6). This association was not detected in PC12 or NIH3T3 cells (lanes 8 and 12). Lower blot, the association of the pTyr317 peptide with Grb2 was detected in all cell lines analyzed (even-numbered lanes). The Tyr317 peptide failed to associate with either STS-1 or Grb2 in all cell lines (odd-numbered lanes).

3.2 STS-1 directly binds ShcA pTyr317 peptide

To determine whether STS-1 binds directly or indirectly to the pTyr317 peptide, we engineered a GST-STS-1 fusion protein that was used in ShcA peptide-binding experiments. Purified GST-STS-1 fusion protein was incubated with five immobilized peptides representing various phosphorylation sites of ShcA and bound proteins were visualized by Coomassie blue staining. Our results show that purified GST-STS-1 binds directly to the pTyr317 peptide (Figure 2, lane 6), thus demonstrating that STS-1 is a genuine ShcA-binding protein. GST-STS-1 did not associate with other ShcA-derived peptides (Figure 2, lanes 2-5).

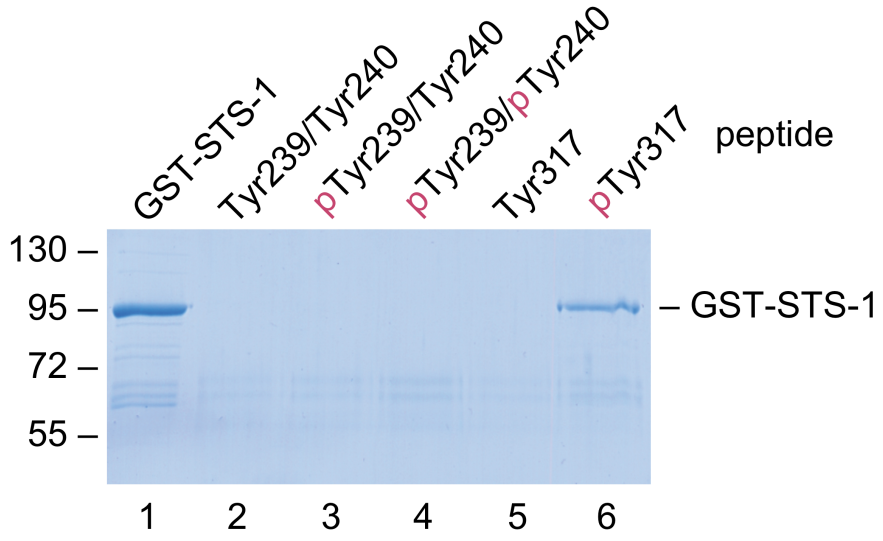

Figure 3.2 STS-1 binds directly to the pTyr317 peptide
GST-STS-1 was purified by affinity chromatography with glutathione-sepharose (Lane 1). Purified GST-STS-1 was then incubated with the various ShcA-derived peptides as indicated at the top of the figure. Only the pTyr317 peptide bound to GST-STS-1, indicating direct association of STS-1 with that ShcA-derived peptide (Lane 6).

3.3 Substitution of Asp314 in the pTyr317 peptide prevents binding of STS-1

To determine the binding specificity of STS-1 for the pTyr317 peptide, mutant pTyr317 peptides containing Ala substitutions were assayed for their ability to associate with STS-1.

In one approach, purified GST-STS-1 was incubated with wild type and Ala-substituted pTyr317 peptides. Bound proteins were resolved by SDS-PAGE and stained with Coomassie to detect the association of GST-STS-1 (Figure 3a). Interestingly, only the pTyr317 peptide containing an Asp314Ala substitution failed to associate with GST-STS-1 (Figure 2, lane 3), whereas all other peptides appeared to bind GST-STS-1 with similar efficiency as the wild type peptide (Figure 3a, lanes 2, 4-8).

In a second approach, a cell-based experiment was performed in which lysates of HEK293 cells were subjected to affinity purification with the wild type and Ala-substituted pTyr317 peptides. Bound proteins were then resolved by SDS-PAGE and analyzed by immunoblotting. As in the previous experiment, the Asp314Ala pTyr317 peptide failed to demonstrate association with STS-1 (Figure 3b, upper blot, lane 2). Similarly as well, the other Ala-substituted peptides appeared to associate with STS-1 in a comparable manner to that of the wild type pTyr317 peptide (Figure 3b, upper blot, lanes 1, 3-7).

To determine how the binding specificity of STS-1 compared to another ShcA-binding protein, Grb2, proteins purified from cell lysates by the wild type and Ala-subsituted pTyr317 peptides were also analyzed by anti-Grb2 immunoblotting. Grb2 failed to associate with the Asn319Ala pTyr317 peptide (Figure 3b, lower blot, lane 6), whereas it was appeared to associate with the wild type and other mutant pTyr317 peptides (Figure 3b, lower blot, lanes 1, 3-7).

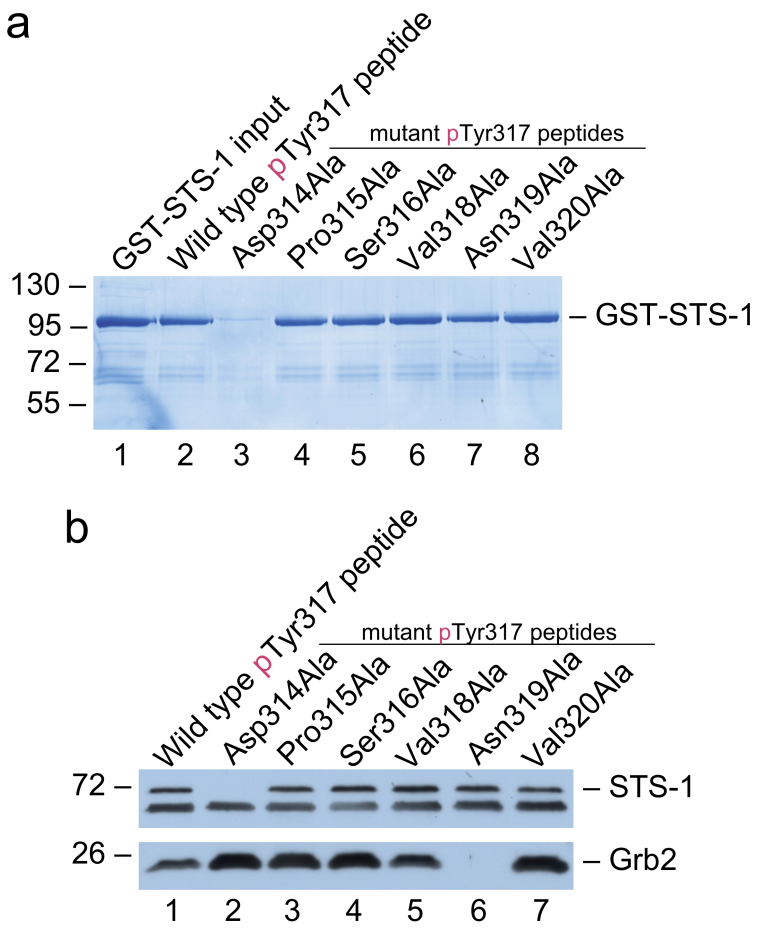

Figure 3.3 Asp314Ala substitution in the pTyr317 peptide prevents association of STS-1
(a) Purified GST-STS-1 (lane 1) was incubated with wild type pTyr317 peptide (lane 2) or variants of the peptide with single Ala substitutions (lanes 3-8). The peptide bearing Asp314Ala mutation failed to associate with GST-STS-1 (lane 3). (b) Lysates from HEK293 cells were probed with wild type and mutant pTyr317 peptides and bound proteins were subjected to immunoblot analysis. Anti-STS-1 immunoblotting confirms that Asp314Ala mutation prevents association of STS-1 with pTyr317 peptide (upper blot, lane 2). These samples were also subjected to anti-Grb2 immunoblotting (lower blot), where Asn320Ala mutation of the pTyr317 peptide blocks association of Grb2 (lane 6).

3.4 The amino-terminal fragment of STS-1 binds pTyr317 peptide

To define the region of STS-1 that binds directly to the peptide, recombinant GST-STS-1 was subjected to limited proteolysis with thermolysin. This generated roughly nine thermolytic peptides, ranging in size from ~80 kDa to 20 kDa (Figure 4, lane 3). The products of the limited proteolysis reaction

were then incubated with the pTyr317 peptide to purify any STS-1 fragments that retained the ability to bind the peptide. A single major 32 kDa fragment (p32) was observed to bind the peptide (Figure 4, lane 4). Other minor bands were not analyzed.

The p32 band was excised from the gel and subjected to Edman degradation. This indicated the first 8 amino-terminal residues of the protein fragment consisted of residues 35-42 of full length STS-1. At 32 kDa, the fragment would consist of residues 35 to approximately 320 of full-length STS-1.

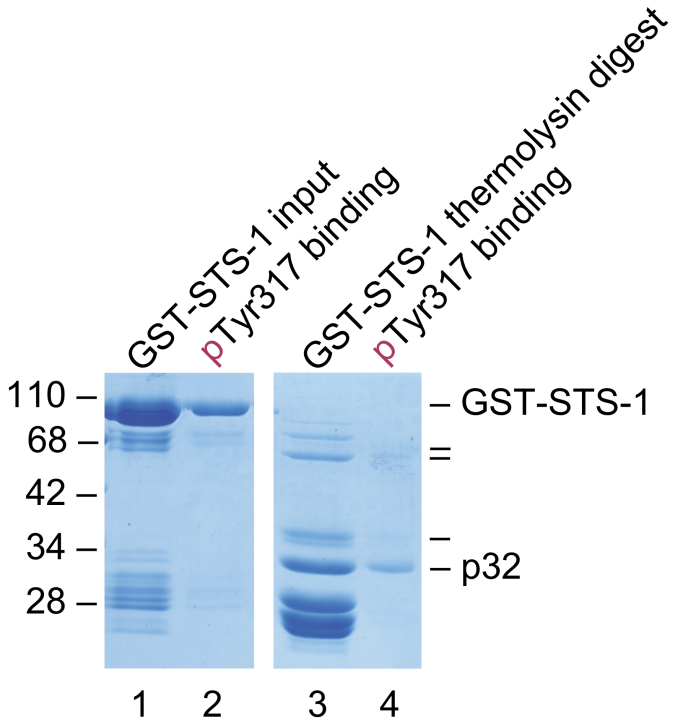

Figure 3.4 Limited proteolysis of STS-1 generates a 32 kDa fragment which binds the pTyr317 peptide.
GST-STS-1 was expressed as a fusion protein in *E. coli* and purified (Lane 1). This fusion protein was pulled down by the pTyr317 peptide in a control experiment (Lane 2). Incubation of GST-STS-1 with thermolysin yielded several products with a variety of molecular weights (Lane 3). When these products were subjected to pull-down with the pTyr317 peptide, a 32 kDa fragment retained binding affinity for the peptide (Lane 4).

3.6 Mutation of either conserved His in domain with homology to 2H phosphoesterase superfamily abolishes binding of STS-1 to pTyr317 peptide

Sequence analysis of STS-1 does not indicate the presence of a domain with ascribed phosphotyrosine-binding activity. Nonetheless, the ability of STS-1 to bind the pTyr317 peptide directly implies that a domain in STS-1 must mediate binding. To further characterize which of the four domains in STS-1 mediates binding to the pTyr317 peptide, HEK293 cells transiently expressing STS-1 or various domain-defective STS-1 constructs were lysed and their lysates were incubated with the pTyr317 peptide. Notably, constructs in which either of the conserved His residues in the domain with homology to the 2HPE superfamily were substituted with Ala, became unable to form a complex with the pTyr317 peptide (Figure 6b, lanes 4 and 5). Importantly, all other constructs retained the ability to associate. All constructs displayed similar levels of expression, as seen in an anti-FLAG immunoblot of the whole cell lysates (Figure 3a). These findings show that the domain of STS-1 with homology to the 2HPE superfamily mediates binding to the pTyr317 peptide.

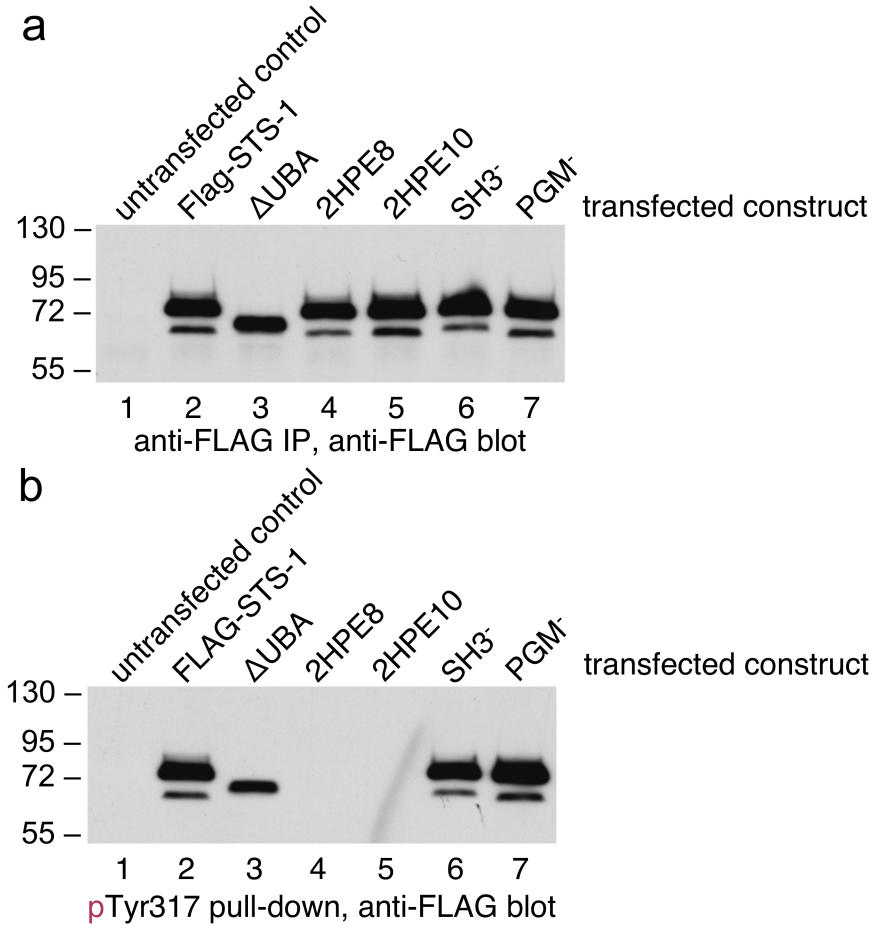

Figure 3.5 Mutation of His126 or His212 precludes association of STS-1 to the pTyr317 peptide FLAG-STS-1 and FLAG-STS-1 constructs with disabled or deleted domains were expressed in HEK293 cells, as noted at top of figures. STS-1 mutations are as follows: ΔUBA, deletion of residues 1-65; 2HPE8, His126Ala; 2HPE10, His212Ala; SH3- Trp295Leu; PGM-, Arg390Ala His391Ala (see figure 7). (a) Expression of each construct was verified by an anti-FLAG blot of anti-FLAG immuonprecipites (lanes 2-7). The identity of the lower molecular weight band is unknown at this time. (b) Lysates of these cells were subjected to affinity purification with the pTyr317 peptide followed by their detection by anti-FLAG immunoblotting. Mutation of either conserved histidine in the region of STS-1 with homology to the 2HPE superfamily generates a protein that is unable to associate with the pTyr317 peptide (lanes 4 and 5).

3.7 STS-1 contains most of the conserved residues found in members of the 2HPE superfamily

Standard sequence analysis of STS-1 reveals three domains, including a ubiquitin-association (UBA) domain, a Src-Homology 3 domain (SH3), as well as a C-terminal tyrosine phosphatase domain related to the phosphoglyclerate

mutase superfamily. Between the UBA and SH3 domains lies a region of protein that shows distant sequence homology to the 2HPE superfamily [102], but up to now has not been ascribed activity (Figure 7).

```
CPD_ARATH 7 DVYSVWALPDEESEPRFKKLMEALRS------EFTGPRFVPHVTVAVSAYLTA 53
AKAP18   86 NYFLSIPITNKEIIKGIKILQNAIIQQDERLAKAMVSDGSFHITLLVMQLLNE 138
STS-1    89 -EYVLYLRPT-GPLAQKLSDFWQQSK--QICGKNKAHNIFPHITLCQFFMCED 137
STS-2    73 -EYALFLCPT-GPLLEKLQEFWRESK--RQCAKNRAHEVFPHVTLCDFFTCED 121

CPD_ARATH   DEAKKMFESACDGL-------KAYTATVDRVSTGTFF-FQCVFLLLQTT---P 95
AKAP18      DEVNIGIDALLELKPFIEELLQGKHLTLPFQGIGTFG-NQVGFVKLAEGDHVN 190
STS-1       SKVDALGEALQTTVS----RWKCKFSAPLPLELYTSSNFIGLFVKEDS----A 182
STS-2       QKVECLYEALKRAGD----RLLGSFPTAVPLALHSSISYLGFFVSGSP----A 166

CPD_ARATH   EVMEAGEHCKNHFN------------------------------CST------ 112
AKAP18      SLLEIAETANRTFQ----------------------------EKGILVGE--- 212
STS-1       EVLKKFAADFAAEA--------------------------ASKTEVHVE-- 205
STS-2       DVIREFAMTFATEASLLAGTSVSRFWIFSQVPGHGPNLRLSNLTRASFVSHYI 219

CPD_ARATH   --------TTPYMPHLSLLYAELT-------EEEKKNAQEKAYTLDSSLDGLS 150
AKAP18      --------SRSFKPHLTFMKLSKSPWLRKNGVKKIDPDLYEKFI---SHRFGE 254
STS-1       --------PHKKQLHVTLAYHFQAS------HLPTLEKLAQNID------VKL 238
STS-2       LQKYCSVKPCTKQLHLTLAHKFYPH------HQRTLEQLARAIP------LGH 260
```

Figure 3.6 Sequence alignment of 2HPE superfamly members
Alignment of cyclic phosphodiesterase from *Arabidopsis thaliana* (CPD_ARATH, UniProt ID 004147), human AKAP18 (UniProt ID Q9POM2), STS-1 (UniProt ID Q8TF42) and STS-2 (UniProt ID P57075) based on Mazmuder *et al.* [102]. These proteins contain a domain that is part of a large superfamily of evolutionarily related proteins with two highly conserved histidine residues, the 2HPE superfamily. Alignment of the STS proteins with other members of this superfamily shows that they possess both hallmark His residues (highlighted in red) as well as several other less highly conserved residues (highlighted in yellow) characteristic of this superfamily. Deviations from conserved residues are bolded in the sequences of STS-1 and STS-2.

STS-1 and STS-2 share an identical domain architecture and nearly 40% amino acid identity. As detailed by Mazmuder *et al.* [102], alignment of the region of STS-1 and STS-2 with homology to the 2HPE superfamily with other members of the superfamily reveals the presence of the two conserved His residues (Figure 5, highlighted in red). Other highly conserved residues are also present in these proteins (Figure 5, highlighted in yellow). Despite lacking

catalytic activity, STS-1, STS-2 and AKAP18 contain nearly all conserved residues.

4. Discussion

ShcA consists of an amino-terminal PTB domain, a central region with multiple tyrosine phosphorylation sites and a carboxy-terminal SH2 domain [60,153]. The PTB domain binds to a variety of activated RPTKs, which then phosphorylate ShcA on tyrosines 239, 240 and 317. The phosphorylation sites in ShcA can then serve as binding sites to recruit Grb2 [145]. Grb2, in turn, activates a variety of signaling pathways as a consequence of its interaction with Gab-1 and SOS, which activate PI3K-Akt [154] and Ras-Erk [68] signaling, respectively. Thus, ShcA can play a direct role in the activation of various signaling pathways, which then regulate a diverse array of cellular activities.

Paradoxically, a variety of RPTKs bind Grb2 directly, which also leads to PI3K-Akt and Ras-Erk activation [155]. We thought it unlikely that this role of ShcA would be maintained through evolution if it were simply a redundant function, which led us to hypothesize that recruitment of ShcA to a given receptor leads to distinct signaling outcomes as compared to receptors that bind Grb2 directly. We found that upon growth factor stimulation, cells expressing ShcA-binding NGFR engaged in greater DNA synthesis than cells expressing a Grb2-binding NGFR variant [146].

To identify novel ShcA-binding proteins that may explain this observation, we designed a variety of peptides based on ShcA-phosphorylation sites for use in affinity purification. Notably, we demonstrated that a peptide based on the Tyr317 phosphorylation site of ShcA associated with the protein STS-1 in a phosphorylation-dependent manner [151]. Further investigation demonstrated that association of endogenously expressed STS-1 and ShcA is responsive to growth factor stimulation [151]. Here, we demonstrate that STS-1 utilizes a domain with homology to the 2HPE superfamily to bind directly to ShcA.

A survey of a mammalian cell lines reveals considerable variability in terms of STS-1 expression (Figure 1). Using the pTyr317 peptide for affinity purification from cell lysates, we detected STS-1 in COS1, A549, 293A, and C6 cells, whereas STS-1 was not detected in PC12 and NIH3T3 cells. This contrasts with the Grb2 association with the peptide, which was detected in all cell lines examined. Interestingly, analysis of STS-1 expression in mice found its presence in all tissues examined [156]. Considering the general role of STS-1 in down-regulating signaling, the loss of STS-1 expression may play a role in the transformation of some cells lines.

Our previous study did not discern whether the interaction of STS-1 with the ShcA-derived pTyr317 peptide was direct or indirect [151]. To address this, we incubated purified GST-STS-1 with the various ShcA-derived peptides to determine if the association is direct (Figure 2). Significantly, we found that that

only the pTyr317 peptide associated with GST-STS-1, thus proving the interaction is direct.

For many protein domains, such as SH2 and PTB domains, binding to tyrosine-phosphorylated proteins is determined primarily by the residues adjacent to the phosphotyrosine [61,157]. Although STS-1 does not contain an SH2 or PTB domain, the fact that it associates specifically with the pTyr317 peptide clearly indicates that STS-1 also shares this property. To characterize the amino acid residues in the pTyr317 peptide that are critical for STS-1 binding, we performed pull-down experiments with pTyr317 peptides containing single Ala substitutions.

In one approach, purified GST-STS-1 was incubated with wild type and Ala-substituted pTyr317 peptides (Figure 3a). Asn314Ala substitution of the pTyr317 peptide prevented the binding of GST-STS-1, whereas all other peptides appear to bind GST-STS-1 with a similar affinity as the wild type peptide. In another approach, HEK293 cell lysates were incubated with the wild type and mutant pTyr317 peptides to purify endogenously expressed STS-1 (Figure 3b). Here, the Asn314Asn pTyr317 peptide also failed to associate with STS-1, whereas all other peptides did bind STS-1. The association of Grb2, which is well described as a ShcA-binding protein, was also analyzed by immunoblot. Grb2 was found to associate with all pTyr317 peptides except one bearing an Asn319Ala substitution. This finding is consistent with others [158,159], whom have found that Grb2 binds to pTyr-X-Asn motifs. These

findings indicate that Grb2 and STS-1 recognize a non-mutual set of residues adjacent to phosphotyrosine on the pTyr317 peptide. However, in order for these proteins to associate with the pTyr317 peptide with specificity, more than a single residue on the peptide must be recognized. It is possible that some of the single Ala substitutions influenced the affinity so minimally that this approach was not able to detect a change in binding affinity when any one particular peptide residue was substituted. Alternatively, residues outside of pTyr-3 to pTyr+3 may be critical mediators of the interaction, as the full-length pTyr317 peptide contains residues from pTyr-7 to pTyr+6 positions.

To determine the region of STS-1 responsible for interaction with the pTyr317 peptide, limited proteolysis experiments were performed to identify the smallest fragment of STS-1 capable of binding the pTyr317 peptide. This led to the purification of p32 (Figure 4). The STS-1 fragment, p32, encompasses the UBA domain, the region with homology to the 2HPE superfamily and the SH3 domain. To further characterize the region of STS-1 responsible for association with the pTyr317 peptide, a series of domain-defective FLAG-STS-1 constructs were overexpressed in HEK293 cells and assayed for association of the construct with the pTyr317 peptide (Figure 5). Remarkably, mutation of either conserved His in the domain with homology to the 2HPE superfamily proved sufficient to abrogate association with the peptide. Taken together these results indicate that a novel phosphotyrosine-binding domain has been detected in STS-1 with distant homology to the 2HPE superfamily.

Sequence analysis of this region of STS-1 does not indicate the absence of any highly conserved residues that are present in other members of the superfamily (Figure 6). This is somewhat surprising, given that this region appears to lack catalytic activity that is associated with most members of the 2HPE superfamily. Alignment of STS-1 and paralog STS-2 with two other 2HPE superfamily members, namely cyclic phosphodiesterase from *Arabidopsis thaliana* and human AKAP18, shows that STS-1 contains nearly all residues that are conserved in this family. However, AKAP18, which stably binds AMP and CMP [160], also contains all residues expected to be involved in catalysis (Figure 6). The observation that STS-1 requires both hallmark histidine residues in order to bind the ShcA-derived pTyr317 peptide would appear to endorse the sequence analysis methods of Mazumder [102].

STS-1 was first discovered in a screen using a Jak2-derived peptide to affinity purify proteins from DA3 cells [156]. The authors identified that, in addition to STS-1, several other signaling proteins also associated with their peptide, including ShcA. However, a direct interaction between STS-1 and ShcA, or any other protein isolated in the screen was not described. Our results suggest ShcA may have linked STS-1 to the Jak2-derived peptide.

Characterization of *STS-1*$^{-/-}$ mice initially revealed no obvious differences from their wild type littermates [156] and it was only when *STS-1* was knocked out in conjunction with its paralog, *STS-2*, that a change in phenotype was observed when compared to wild type littermates [161]. These *STS-1/2*$^{-/-}$ mice

display hyperactivation of a variety of signaling proteins following T cell receptor activation, as well a marked increase in T cell cytokine production [161], indicating the two proteins have overlapping effects at least in some cell types. Further investigations of *STS-1*$^{-/-}$ mice later revealed hyperactive T-cells, platelets, and mast cells when compared to their wild-type littermates [162–165]. Orthologs of STS-1 are found in organisms as divergent as *C. elegans* and *H. sapiens* and in mice this protein was detected in all tissues examined [156]. These findings suggest that STS-1 serves a critical role in organismal fitness, albeit one that may be hard to detect in the confines of a laboratory.

Dikic *et al.* showed that an intact UBA and SH3 domain are required for STS-1 to inhibit receptor endocytosis, a principal mechanism for limiting receptor signaling [166]. To do this, STS-1 uses its UBA domain to bind ubiquitinated receptors while the SH3 domain recruits c-Cbl. The authors propose that this dynamic may alter the dynamics of endocytic sorting and thus retard ubiquitin-mediated internalization of the receptor [166]. STS-1 is also a potent tyrosine phosphatase and several signaling proteins have been shown to be depohosphorylated by it, including Zap-70, Src, EGFR [103], and Syk [104,156]. Dephosphorylation of these proteins results in their decreased activity and is thus appears to be a direct mechanism by which STS-1 acts to suppress signaling. That STS-1 may stably phosphotyrosine residues and also be a tyrosine phosphatase is not a unique activity. The SH2 domain-containing tyrosine phosphatase, SHP1, is well characterized in this regard [167,168].

The emergence of RPTKs coincided with that of phosphotyrosine-binding domains like SH2 and PTB domains some 900 million years ago [169]. In fact, it is proposed that the development of phosphotyrosine-mediated signaling was essential for the evolution of multicellularity in metazoans [147], a group that also emerged around 900 million years ago. Currently, SH2 and PTB domains, as well as the C2 domain from PKCδ [49], are the only domains characterized as having the capacity to bind proteins in a phosphotyrosine-dependent manner. It appears a single ancestral gene was repeatedly duplicated and mutated to generate the roughly 110 SH2-domain containing proteins that are present in the human genome [169]. PTB domains also share a common genetic origin. Some PTB domains exhibit phosphotyrosine-dependent binding to NPXpY motifs, although numerous others simply bind unphosphorylated NPXY motifs. These observations demonstrate the considerable evolutionary plasticity of protein domains. Alternatively, there are likely to be many other protein domains whose functions diverged overtime to permit phosphotyrosine-dependent binding, such as the C2 domain of PKCδ [49]. Of course, due to a lack of known sequence homology, it has proven difficult to identify these types of domains *en masse* despite their potential significance in signal transduction pathways. Although not evolutionarily related to PTB or SH2 domains, phosphotyrosine-binding proteins like STS-1 are critical participants in RPTK signaling.

Many protein domains involved in protein-protein interactions primarily interact with only a relatively short peptide motif [170]. For instance, SH2 domains, which confer the ability to associate with tyrosine-phosphorylated proteins, selectively bind a phosphotyrosine residue and between three and six adjacent residues carboxy-terminal to the phosphorylated tyrosine residue [48]. Similarly, SH3 domains recognize poly-proline motifs (PxxP) within a short peptide sequence [171,172]. As mentioned, PTB domains also bind to their partners in this fashion, preferring NPXY motifs [173]. Thus, because many protein-protein interaction domains primarily recognize only a short peptide segment of a larger protein, chemically synthesized peptides can be exploited to identify novel protein-protein interactions [174].

Most members of the 2HPE superfamily are enzymes that are involved in tRNA splicing, phosphonate metabolism and intron removal, such as 2', 3' cyclic phosphodiesterases [175] and RNA ligases [176]. Other members, such as the PKA anchoring protein AKAP18, have been shown to bind small molecules like AMP [160]. These activities are all mediated by the presence of a phosphate group on the bound molecule. The binding of a phosphorylated tyrosine group by the ShcA-binding domain in STS-1 therefore seems to be a plausible capacity of this family of domains. To our knowledge, this is the first example of a member of the 2HPE superfamily to mediate a protein-protein interaction. The discovery of a novel domain that mediates phosphotyrosine-

dependent binding represents a potentially significant contribution to the field of tyrosine kinase signaling.

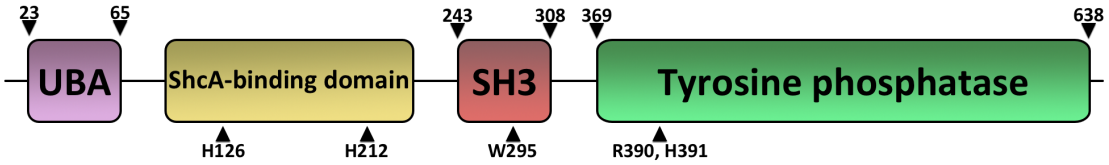

Figure 3.7 Domain architecture of STS-1 and sites of point mutations
STS-1 contains four domains that are involved in signal transduction. The approximate confines of each domain are indicated at top of figure. As predicted by their primary structures, the UBA domain binds to ubiquitin, while the SH3 domain mediates protein-protein interactions. The tyrosine phosphatase domain is related to the PGM superfamily and dephosphorylates a variety of signaling proteins. Based on our findings, we propose the region of STS-1 related to the 2HPE superfamily be termed the ShcA-binding domain. Bottom of figure, the location of point mutations in FLAG-STS-1 constructs with defective domains is shown.

Chapter 3, in full, is currently being prepared for submission for publication of the material. Swarts, Spencer; van der Meulen, Talitha; Fischer, Patel, Jesal; van der Geer, Peter. Identification of phosphotyrosine- and ShcA-binding domain in STS-1. The dissertation author is author and investigator of this material.

Chapter 4. Sts-1 regulates ShcA tyrosine phosphorylation and Akt activation

Abstract

STS-1 is tyrosine phosphatase that participates in signal transduction downstream of protein tyrosine kinases. Using peptides derived from ShcA, we recently demonstrated that STS-1 binds to the Tyr317 region of ShcA in a phosphorylation-dependent manner. Here, we show that STS-1 can also directly dephosphorylate ShcA *in vitro* in a time-dependent manner. In a cell-culture model, we also demonstrate that cells lacking STS-1 display higher levels of ShcA tyrosine phosphorylation following EGF stimulation. STS-1-deficient cells also display enhanced activation of Akt, but not Erk.

1. Introduction

Protein tyrosine kinases (PTKs) transmit signals by phosphorylating particular tyrosine residues on intracellular proteins. In many cases, this generates binding sites for SH2 or PTB domain-containing proteins, which bind phosphorylated tyrosine groups in the context of the adjacent amino acids [7]. Recently, we identified STS-1 a novel ShcA-binding protein [151]. Specifically, using phsophopeptides based on known phosphorylation sites, we found that STS-1 binds the Tyr317 region of ShcA in a phosphotyrosine-dependent manner. Moreover, STS-1 uses a domain with distant homology to the 2H phosphoesterase (2HPE) superfamily to associate with phosphorylated ShcA

[manuscript in progress]. This domain has since been termed the “ShcA binding” domain.

Several other tyrosine phosphatases also contain domains that permit phosphotyrosine binding. The tensins contain tandem SH2 and PTB domains, whereas the SHP proteins contain tandem SH2 domains. These domains play a critical role in localization to particular intracellular locations, where the tyrosine phosphatase domains encounter their physiologically relevant substrates [177,178]. Interestingly, SHP1 substrates have been identified that also bind to SHP1 stably. For instance, SHP1 can both stably bind and dephosphorylate EGFR [179]. Because STS-1 contains a tyrosine phosphatase domain, as well as a phosphotyrosine-binding domain, we wondered if STS-1 might have phosphatase activity toward proteins that it binds stably. To address this question, a variety of *in vitro* and cell-based assays were performed to evaluate ShcA tyrosine phosphorylation in relation to STS-1.

Here, we provide evidence that STS-1 may be a physiologically relevant tyrosine phosphatase of ShcA. In an *in vitro* phosphatase assay, purified STS-1 dephosphorylated ShcA in a time-dependent manner. Furthermore, STS-1-deficient cells demonstrate higher levels of ShcA tyrosine phosphorylation following EGF stimulation than do control cells. When examining signaling downstream of ShcA, enhanced Akt, but not Erk, activation was found in stimulated cells lacking STS-1. These results suggest that STS-1 can directly

dephosphorylate tyrosine residues in ShcA and that this activity consequently modulates downstream signaling activity.

2. Materials and methods

2.1 Reagents

EGF and the anti-phosphotyrosine monoclonal antibody 4G10 were purchased from Fisher Scientific (Waltham, MA). A polyclonal anti-STS-1 serum was purchased from Abcam (Cambridge, MA). A polyclonal anti-serum was raised against a GST-ShcA-SH2 domain fusion protein [152]. Anti-Erk1/2, anti-phsopho-Erk (Thr202/Tyr204), anti-Akt and anti-phospho-Akt (Thr308) were purchased from Cell Signaling Technologies (Danvers, MA). Protein A-horseradish peroxidase (HRP) and sheep anti-mouse-HRP were purchased from Sigma-Aldrich (St. Louis, MO). Protein A-agarose was purchased from Repligen (Waltham, MA).

2.2 Cell lines, stimulation and lysis

HEK293 and A549 cells were grown on 10 cm tissue culture dishes in DMEM supplemented with 10% FBS. Prior to stimulation, cells were starved for 16 hours in DMEM with 20 mM HEPES, pH 7.2. Control cells and cells stimulated with 50 ng/mL EGF were lysed as follows. Tissue culture dishes were rinsed twice with 5 mL 10 mM Tris, 150 mM NaCl (TBS), before the addition of 1mL 50mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$ 1mM EGTA, 100 mM NaF, 10 mM sodium pyro- phosphate, 500 M

sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin (PLC lysis buffer). Lysates were clarified by centrifugation at 10,000 rpm for 20 minutes at 4°C.

2.3 Immunoprecipitation

To obtain anti-ShcA immunoprecipitates, cell lysates were incubated with 2 μL polyclonal anti-ShcA antiserum for 1 hour on ice. 100 μL 10% ProteinA-agarose was then added and samples were placed on a rocker at 4°C for 1 hour and then washed four times in PLC lysis buffer. Samples were either stored for use in further experiments as described below or sample buffer was added.

2.4 Immunoblotting

Sample were first resolved by SDS-PAGE and then transferred to PVDF membranes. Membranes were blocked for 1 hour at room temperature in 10 mM Tris/Cl, pH 7.4, 150 mM NaCl, 0.2% Tween 20 (TBS-T) containing 5% dried milk and incubated with antisera in TBS-T with 5% milk for 1 hour at room temperature. Anti-Erk, anti-pErk, anti-Akt and anti-pAkt antibodies were diluted per manufacturer's recommendation, whereas anti-ShcA and anti-STS-1 antibodies were diluted 1:200 and anti-phosphotyrosine serum was diluted 1:5 in respective blocking solution. Blots were washed twice for 10 min with TBS-T and twice for 5 min with 10mM Tris/Cl, pH 7.4, and 150mM NaCl (TBS). Membranes were then incubated for 30 min with horseradish peroxidase-proteinA or horseradish peroxidase goat anti-mouse (Bio-Rad) diluted 1:10,000

in TBST and then washed as before. Reactive proteins were visualized by ECL (Amersham Pharmacia Biotech). For immunoblotting with anti-phosphotyrosine, anti-Erk, anti-Akt and anti-pAkt antibodies, membranes were blocked with TBS-T containing 5% bovine serum albumin followed by an incubation with the antiserum diluted (according to manufacturer's recommendation) in TBST with 5% bovine serum albumin.

2.5 *In vitro* STS-1-mediated dephosphorylation of ShcA

Anti-ShcA immunoprecipitates were collected from HEK293 cells stimulated with EGF for 5 minutes. Recombinant GST-STS-1(380-649) fusion protein was generated by cloning cDNA for STS-1 into the pGEX-4T2 plasmid. Expression of GST-STS-1 was induced with 100 μM IPTG for 6 hours at 37 °C. Bacteria were lysed by sonication in phosphate-buffered saline (PBS) containing 1 mM DTT, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1% Triton X-100. Lysates were clarified by centrifugation for 10 min at 10,000 g at 4 °C. Fusion proteins were then purified by binding to glutathione-agarose. Glutathione-agarose beads were collected by centrifugation and washed 4 times with PBS containing 1% Triton X-100, 1mM DTT, 1mM benzamidine. Bound protein was eluted with glutathione and dialyzed. Fusion proteins were stored at 4 °C in PBS containing 1% Triton X-100, 1 mM DTT, 1 mM benzamidine, 0.02% sodium azide, and 10% glycerol. Purified GST-STS-1 (~0.1 mg/mL final concentration) was incubated with anti-

ShcA immunoprecipitates. Reactions took place at 4°C on a rocker for the indicated times, at which time sample buffer was added to quench the reaction.

2.6 STS-1 constructs and transfection

Wild type and mutant STS-1 constructs were cloned into pcDNA3.1 and sequenced to confirm fidelity. For transfection, 10^6 HEK293 cells were seeded on 10 cm tissue culture dishes. 24 hours later cells were transfected with 3 µg DNA/plate using Lipofectin according to the manufacturer's instruction (Thermo Fisher, Waltham, MA). Control cells were transfected with empty pcDNA3.1 vector. 48 hours after transfection cells were starved and then subjected to EGF stimulation and lysed.

2.7 Genetic ablation of STS-1

To generate cells deficient in STS-1, we utilized the Crispr-Cas9 system. In short, pX330 was digested with BbsI and annealed oligonucleotides consisting of 5'- AAA CAG TCC GCT CGG CAT GGC TGC -3' and 5'- CAC CGC AGC ACT GCC GAG CGG ACT -3' were ligated into the digested vector to generate an *STS-1*-targeting Cas9 construct, see [180] for details. This construct was transfected into A549 cells, along with pEGFP. Fluorescing cells were then collected by FACS and individual cells were placed in 96-well plates. Cells displaying growth were then analyzed for the presence of STS-1 by immunoblotting. Finally, cells lacking detectable STS-1 were sequenced to confirm the presence of a frame shift mutation.

3. Results

3.1 STS-1(380-649) dephosphorylates ShcA in vitro

ShcA is an adaptor protein that is recruited to activated RPTKs where it is phosphorylated on residues 239, 240, and 317. As a ShcA-binding protein that contains a tyrosine phosphatase domain, we wondered if STS-1 might also dephosphorylate ShcA. To test this, we expressed the tyrosine phosphatase domain of STS-1 as a GST fusion protein (GST-STS-1(380-649)), which was purified from *E. coli* and incubated with immobilized phosphorylated ShcA for various amounts of time. Figure 1A shows equal amounts of the three ShcA isoforms were immobilized for the time course assay. Electrophoretic mobility shift causes the phosphorylated form of ShcA from stimulated cells to migrate more slowly than the unphosphorylated form. GST-STS-1(380-649) appears to cross react with the anti-ShcA antibodies and appears as a 55 kDa band in lanes 2 through 4. After the indicated amounts of time, tyrosine phosphorylation of ShcA was analyzed by western blotting with anti-phosphotyrosine antibodies. Figure 1B shows abundant tyrosine phosphorylation of ShcA in the absence of STS-1 (Lane 1). Interestingly, incubation of GST-STS-1 (380-649) with immobilized ShcA leads to a decrease in ShcA tyrosine phosphorylation in a time-dependent manner. These results show that STS-1 can directly dephosphorylate phosphotyrosine residues in ShcA.

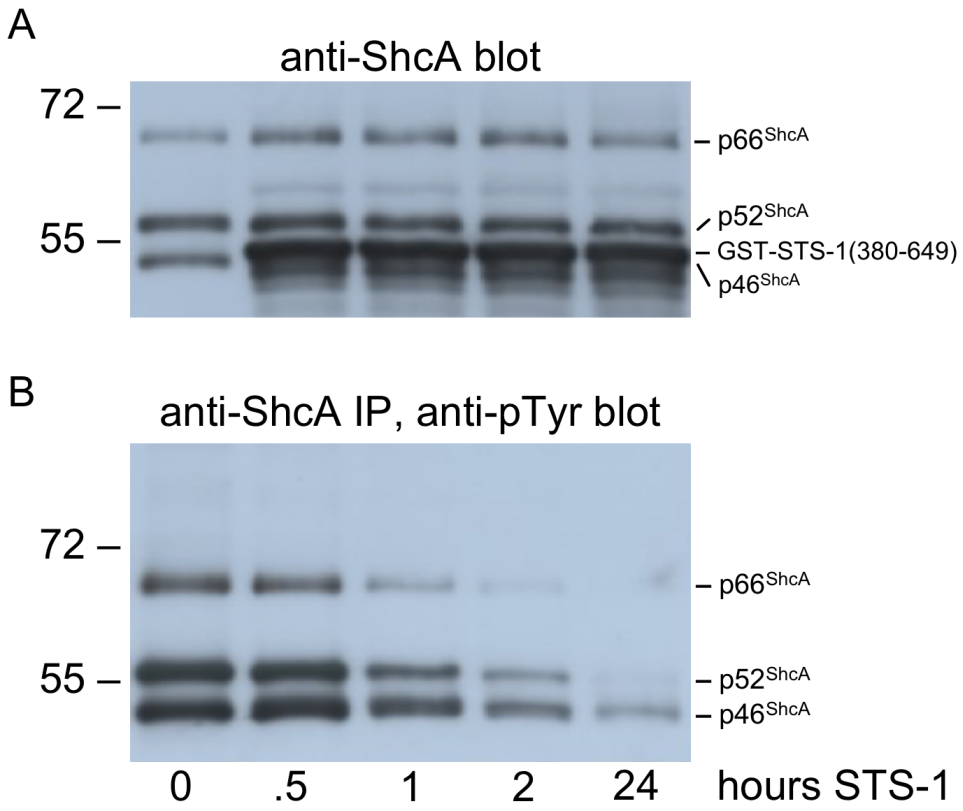

Figure 4.1 The tyrosine phosphatase domain of STS-1 dephosphorylates ShcA in vitro
The tyrosine phosphatase domain of STS-1 dephosphorylates ShcA *in vitro*. Recombinant GST-STS-1(380-649) was incubated with immobilized, phosphorylated ShcA for the period of time indicated at bottom of figure. (A) Anti-ShcA immunoblot reveals that roughly equal amounts of the three ShcA isoforms were isolated in all experiments. GST-STS-1(380-649) appears to cross react with the anti-ShcA antibodies and is present in lanes 2-5 at ~55kDa. (B) Tyrosine phosphorylation of the three ShcA isoforms decreases over a period of 24 hours, indicating that STS-1 can directly dephosphorylate ShcA.

3.2 Overexpression of STS-1 or STS-1 domain-defective mutants does not affect Erk or Akt activation in STS-1$^{-/-}$ cells

The ability of STS-1 to dephosphorylate ShcA suggests that STS-1 may regulate signaling downstream of ShcA. To test this, HEK293 cells overexpressing STS-1 or one the domain-defective STS-1 constructs were stimulated with EGF and compared to control cells lacking STS-1

overexpression. In control cells, EGF stimulation resulted in enhanced Erk phosphorylation, which was undetectable prior to stimulation (compare lanes 1 and 2). In these cells, Akt phosphorylation was present in the absence of EGF (Lane 1) and remained at the same level upon addition of EGF (Lane 2). Erk and Akt phosphorylation was not affected by overexpression of STS-1 (Lane 3), nor any of the other domain-defective STS-1 constructs (Lanes 4-8).

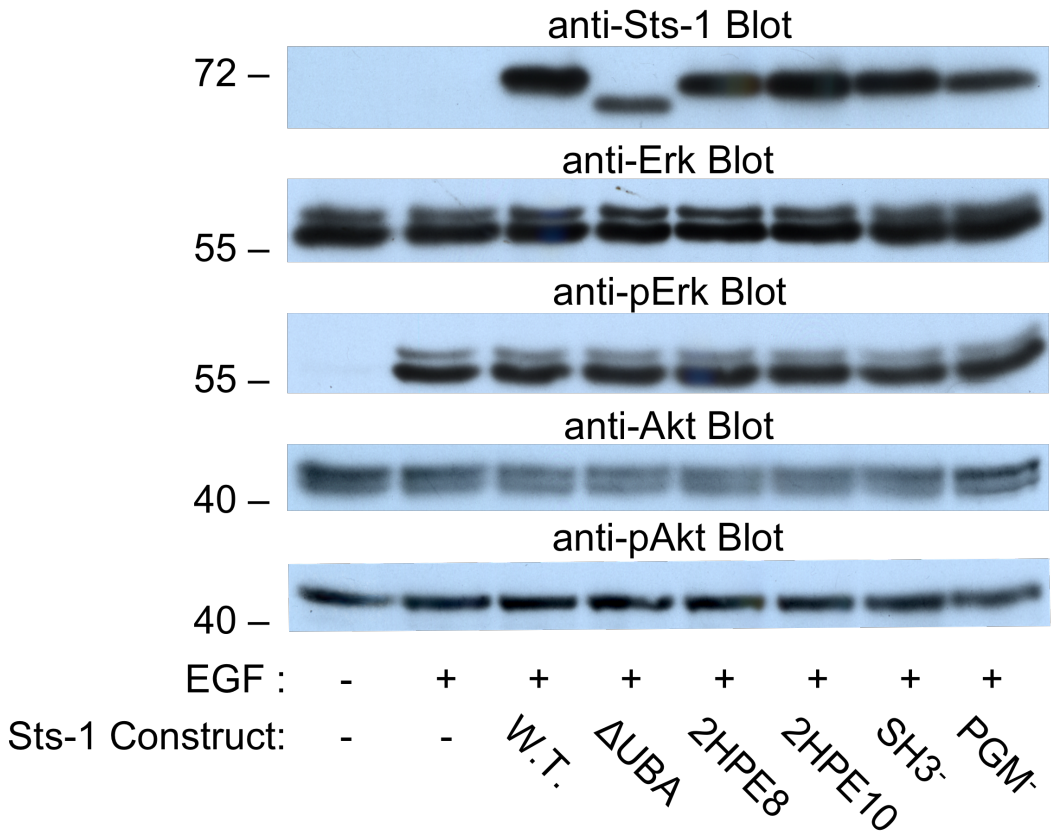

Figure 4.2 Overexpression of wild type or domain-defective STS-1 constructs does not affect Erk or Akt activation
HEK293 cells were transiently transfected with wild-type or domain-defective *STS-1* constructs. EGF stimulation resulted in robust phosphorylation of Erk, but did not enhance Akt phosphorylation in control cells (Lanes 1 & 2). Overexpression of wild-type STS-1 does not appear to alter the phosphorylation of Erk or Akt in these cells (Lane 3). Additionally, expression of other STS-1 constructs does not affect Erk or Akt phosphorylation (Lanes 4-8).

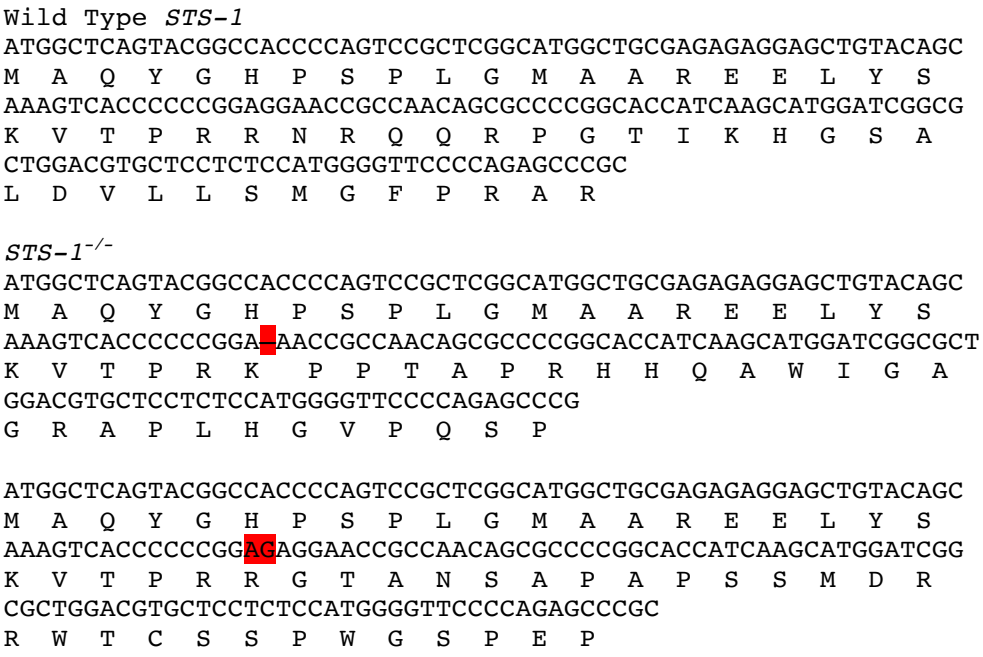

Figure 4.3 Analysis of CRISPR-Cas9-induced *STS-1*$^{-/-}$ A549 cells
At top, the sequence of exon 1 of wild type *STS-1* and the amino acid sequence of the translated DNA. Middle and bottom, sequences show mutations in *STS-1*$^{-/-}$ cells. Due to the diploid nature of the cells, two mutations are required to functionally knock out *STS-1*. In the first allele, a two base pair deletion generates a frameshift mutation. In the second allele, a two base pair insertion generate another frameshift mutation. These will lead to the expression of mutated protein that will contain a pre-mature stop codon. Insertion and deletions are highlighted in red.

3.3 CRISPR-induced knockout of STS-1

To determine the cellular role of STS-1, STS-1-deficient A549 cells were generated for comparison against wild type A549 cells. Figure 2, top, shows the DNA sequence of wild type *STS-1* beginning from the ATG start codon through the remainder of the first exon. To generate STS-1-deficient cells, two mutations were required in the A549 cell line to disable each *STS-1* gene. Figure 2, middle and bottom, shows the DNA sequence of each disrupted gene in the STS-1-

deficient cells. One mutation involved a deletion of bases 77 and 78 of exon 1. The other mutation was an insertion of two bases, AG, between bases 75 and 76 of *STS-1* exon 1.

3.4 STS-1-deficient cells display higher levels of tyrosine phosphorylation on ShcA and enhanced levels of Erk Activation

While the previous *in vitro* assay demonstrated that STS-1 can dephosphorylate ShcA, we wanted to determine whether STS-1 does regulate the phosphorylation of ShcA in the context of cells grown in tissue culture. To do this, we engineered STS-1-deficient A549 cells as described above and compared them to wild type A549 cells. In the absence of stimulation, both wild type and STS-1-deficient cells display low levels of ShcA tyrosine phosphorylation (Figure 3, bottom, lanes 1 and 3). Upon stimulation with EGF, both wild type and STS-1-deficient cells respond with large increase of ShcA tyrosine phosphorylation. However, STS-1 deficient cells show a much greater increase of ShcA tyrosine phosphorylation than do the wild type cells. Interestingly, the ShcA p52 isoform seems to display the greatest increase in tyrosine phosphorylation in response to EGF when comparing the wild type cells to the STS-1-deficient cells. Examination of Erk and Akt activation reveals a large increase in Akt activation in the STS-1-deficient cells, whereas Erk activation remained unchanged.

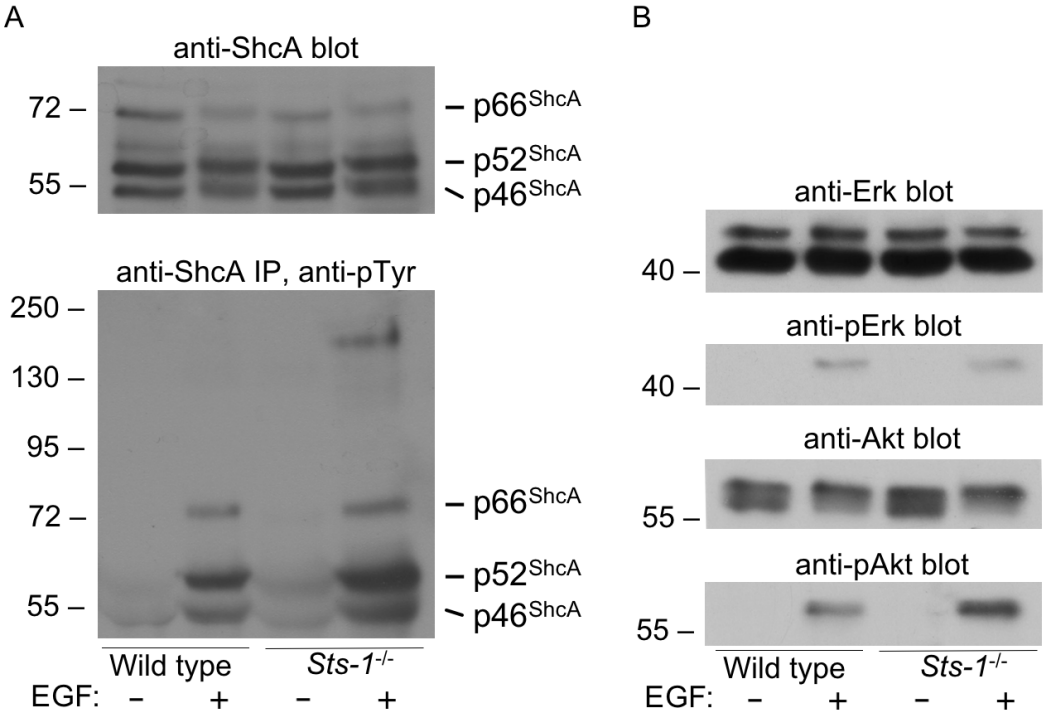

Figure 4.4 STS-1-deficient cells display higher levels ShcA and Akt phosphorylation
Wild type and *STS-1*$^{-/-}$ A549 cells were grown to confluence and placed on minimal media prior to lysis. Cells were either left unstimulated (Lanes 1 and 3) or stimulated with 50 ng/mL EGF for 5 minutes (Lanes 2 and 4). At top, whole cell lysates were probed for the presence of ShcA. (A) Equivalent amounts of the ShcA isoforms (p46Shc, p52Shc, p66Shc) are present across all lanes. (B) In response to EGF stimulation, the STS-1-deficient cells respond with a greater level of tyrosine phosphorylation on p52Shc isoform as compared to the stimulated wild type cells. The other ShcA isoforms appear to display enhanced tyrosine phosphorylation in the stimulated STS-1-deficient cells as compared the stimulated wild type cells, albeit not as much as the p52Shc isoform.

4. Discussion

Recently, we demonstrated that STS-1 binds directly to the phosphorylated Tyr317 region of ShcA using a novel phosphotyrosine-binding domain with distant homology to the 2HPE superfamily [151], [manuscript in progress]. STS-1 is also a tyrosine phosphatase that has been shown to target tyrosine-phosphorylated proteins such as Syk, Fyn [181], Src, and Zap-70 [103], EGFR [103,182], and c-Cbl [182]. These findings led us to investigate the role

STS-1 may play in modulating the tyrosine phosphorylation ShcA and the proteins activated downstream of ShcA, specifically Erk and Akt.

To determine if STS-1 could act as on ShcA directly as a phosphatase, an *in vitro* assay was performed with the purified tyrosine phosphatase domain of STS-1 and immobilized ShcA (Figure 1). To ensure high levels of tyrosine phosphorylation, cells were stimulated with EGF prior to ShcA immunoprecipitation. Analysis of ShcA tyrosine phosphorylation revealed a time-dependent decrease, such that after 24 h at 4°C it was undetectable. These results suggest STS-1 can directly dephosphorylate ShcA. However, in the context of intracellular signaling, these two proteins are amongst – and potentially interact with – thousands of others in the cellular milieu. So, to gain further insight into the cellular role of STS-1, we employed cell-based assays that took this property into account.

A variety of approaches to alter STS-1 expression levels, such as under- and overexpression, in cells and then analyze the aforementioned effects failed to reveal meaningful differences with control cells. Figure 2 shows a representative experiment in which wild type STS-1 and other domain-defective STS-1 constructs were overexpressed in HEK293 cells. In this experiment, overexpression of STS-1 fails to affect Erk or Akt activation following EGF stimulation. Additionally, the other domain-defective STS-1 constructs also fail to affect Erk or Akt activation. Efforts to analyze the role of STS-1 using RNAi to deplete cells of STS-1 had similar results (results not shown). The inability to

identify differences in cells overexpressing STS-1 may have been the result of endogenous STS-1 expression in control cells as STS-1 is expressed ubiquitously [156]. To overcome these issues, we employed a CRISPR-Cas9-based system to completely eliminate STS-1 expression through genetic disruption. A construct targeting exon 1 of *STS-1* successfully introduced frameshift mutations into the two *STS-1* genes present in A549 cells (Figure 2).

Comparison of wild type A549 cells with *STS-1*$^{-/-}$ A549 cells failed to reveal differences in terms of cell morphology, growth rate, migration, and extravasation under a variety of conditions (results not shown). Interestingly though, cells completely lacking STS-1 expression show significantly more ShcA tyrosine phosphorylation following EGF stimulation (Figure 4a). Additionally, examination of proteins activated downstream of ShcA revealed enhanced Akt, but not Erk, activation (Figure 4b).

STS-1 was originally isolated from DA-3 cells in which a Jak2-derived phosphopeptide was used as an affinity reagent for protein isolation [156]. Interestingly, this approach also identified ShcA in complex with the Jak2-derived peptide. We recently described STS-1 as a ShcA-binding protein [151], [manuscript in progress]. These findings suggest ShcA may act as bridge that allows STS-1 to be recruited to Jak2 and, presumably, other RPTKs to which ShcA binds.

STS-1 contains a variety of protein domains that allow it to participate in signal transduction. STS-1 contains an amino-terminal UBA domain that binds

ubiquitin [183], an SB domain (related to 2HPE superfamily) that binds ShcA in a phosphorylation-dependent manner [151], an SH3 domain that binds the E3 ubiquitin ligase c-Cbl [166,183] and a carboxy-terminal tyrosine phosphatase domain that has activity against a variety of signaling proteins [103–105,181]. Our results suggest that STS-1 may be a *bona fide* ShcA tyrosine phosphatase that plays a role in modulating signaling from RPTKs via its interaction with ShcA, by regulating Akt activation.

Interestingly, unrelated tyrosine phosphatases such as SHP1 and the tensins also contain phosphotyrosine-binding domains [90]. In fact, SHP1 uses its SH2 domain to bind a variety of targets, some of which it also dephosphorylates [179]. This is similar to STS-1, which binds tyrosine phosphorylated ShcA using the SB domain and dephosphorylates ShcA using a tyrosine phosphatase domain. This arrangement may enhance signaling fidelity by ensuring that these phosphatases are positioned near their substrates, thereby enhancing their specificity.

ShcA contains three tyrosines (239, 240, 317) that are phosphorylated following growth factor stimulation and STS-1 binds Tyr317 region of ShcA. It will be important to determine specifically which tyrosines are dephosphorylated by STS-1. Our anti-phosphotyrosine antibodies do not allow the discernment of which specific tyrosines are phosphorylated. Therefore, judicious selection of antibodies may allow one to tease apart the order (if any) of STS-1-mediated ShcA dephosphorylation.

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