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Regulation of PDGFRβ signaling

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Abstract

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Platelet-derived growth factor (PDGF) isoforms, which bind to closely related a- and b-tyrosine kinase receptors, induce migration, proliferation, survival and differentiation of mesenchymal cells. They signal by the active receptor attracting Src homology 2 (SH2) domain containing proteins, which subsequently initiate a set of signaling pathways. The aim of this thesis was to elucidate regulatory mechanisms involved in PDGFRb signaling.

In the first two projects we investigated the roles in downregulation of PDGFRb of two related adaptor proteins, i.e. ALG-2 interacting protein X (Alix) and His-domain containing protein tyrosine phosphatase (HD-PTP) functions of. We found that Alix and HD-PTP influence ubiquitination of PDGFRb following PDGF stimulation, by affecting the E3 ligase c-Cbl. Alix enhances complex formation between c-Cbl and PDGFRb, increases c-Cbl phosphorylation and decreases its stability. Interestingly, while both HD-PTP and Alix participate in degradation of PDGFRb, only Alix affects receptor internalization. Moreover, we demonstrated that absence of HD-PTP promotes cell proliferation. In conclusion, we suggest that both Alix and HD-PTP are important adaptor proteins in regulation of PDGFRb downregulation, although the observed differences between their actions suggest that Alix and HD-PTP exert their functions via different mechanisms.

The third study explored the importance of tyrosine residue 857 in the activation loop of PDGFRb. We report that, *in vitro* the tyrosine residue 857 to phenylalanine (Y857F) mutant receptor kinase activity is diminished while *in vivo* it does not affect the phosphorylation of PDGFRb. The phosphorylation pattern of PDGFRb revealed that most sites in the Y857F mutant receptor were phosphorylated similarly as in the wild-type receptor. However, tyrosine residue 771 was found to be hyperphosphorylated in the Y857F mutant receptor. This may be due to defective phosphorylation and activation of SHP-2, since it has been shown to dephosphorylate the receptor at Y771. In addition, activation of the Erk1/2 and Akt pathways was defective downstream of the Y857F mutant receptor. Interestingly, the Y857F mutant receptor was able to mediate cell migration, but not proliferation.

The last study investigated a role of the tyrosine kinase Fer in PDGF signaling. We showed that Fer interacted with and was activated by PDGFRb in a ligand-dependent manner. In cells depleted of Fer, receptor phosphorylation was decreased and phosphorylation of Stat3 was abolished, whereas Stat5, Erk1/2 and Akt were activated normally. Colony formation in soft agar was abolished in cells depleted of Fer, but no effect was seen on cell proliferation and migration. Since Stat3 has been shown to be involved in transformation, we speculate that phosphorylation of Stat3 in Fer-depleted cells, affects the ability of cells to form colonies.

Keywords: PDGF HD-PTP Alix Cbl Ub Fer Y857 Y771downregulation degradation ubiquitination activation loop

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For Aga, Staś, Tosia
and
my wonderful Family

... they will not convince us that white is white and black is black!
- former Prime Minister of Poland, Jarosław Kaczyński

„A real man is recognized by the way he finishes, not by the way he starts off."
- former Prime Minister of Poland, Leszek Miller

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Lennartsson J, Wardega P, Engström U, Hellman U, Heldin C-H (2006) Alix facilitates the interaction between c-Cbl and platelet-derived growth factor beta-receptor and thereby modulates receptor down-regulation. *J Biol Chem.* 22;281(51):39152-8

II Wardega P, Jurek A, Engström U, Heldin C-H, Lennartsson J (2010) HD-PTP is important for platelet-derived growth factor beta- receptor downregulation and mitogenicity. *Manuscript*

III Wardega P, Heldin C-H, Lennartsson J (2005) Mutation of tyrosine residue 857 in the PDGF β-receptor affects cell proliferation but not migration. *Manuscript*

IV Lennartsson J*, Wardega P*, Ma H, Pelka K, Engström U, Hellberg C, Heldin C-H (2009) The Fer tyrosine kinase is necessary for platelet-derived growth factor-BB-induced Stat3 phosphorylation and colony formation in soft agar. *Manuscript*
**The first two authors contributed equally to this work*

Contents

Abbreviations

Alix	ALG-2-interacting protein-X
AP-2	Adaptor protein 2
ATP	Adenosine triphosphate
Cbl	Casitas B-lineage lymphoma
DAG	Diacylglycerol
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Erk	Extracellular regulated kinase
ESCRT	Endosomal sorting complex for transport
Fer	Fps/Fes related tyrosine kinase
GEF	Guanine nucleotide exchange factor
HD-PTP	His-domain-containing protein tyrosine phosphatase
Hrs	Hepatocyte growth factor–regulated tyrosine kinase substrate
MAPK	Mitogen activated protein kinase
MVB	Multivesicular bodies
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor

PH	Pleckstrin homology
PI3K	Phosphoinositide-3’-kinase
PLC	Phospholipase C
PtdIns	Phosphatidylinositol
PTP	Protein tyrosine phosphatase
PTP1B	Protein tyrosine phosphatase 1B
RasGAP	Ras GTPase activating protein
RING	Really interesting new gene
RTK	Receptor tyrosine kinases
SH2	Src homology 2
SH3	Src homology 3
Src	Rous sarcoma virus oncogene
STAT	Signal Transducers and Activators of
TC-PTP	Transcription
Tsg101	Tumor susceptibility gene 101
Ub	Ubiquitin
Vps	Vesicular protein sorting

Introduction

The complexity of the internal cell machinery can be compared with nothing that is man-created. Feed-back, feed-forward loops, both negative and positive, pathway cross-talks, scaffolding, activation, deactivation, gene transcription, mRNA translation, posttranslational modifications, and multiprotein complexes at work, control and drive this ATP-fuelled life form. One core function of a cell within a multicellular organism is to communicate with the outside world and one of way to converse with other cells is by releasing soluble signaling molecules, for example growth factors. Communication with other cells within a tissue and between tissues is essential for many biological processes, such as cell survival, proliferation, migration or differentiation. Signaling enables a cell to influence other cells and the environment, or itself in autocrine manner. A simplistic description of a growth factor signal can be separated into several steps; a growth factor is secreted from a donor cell, bound by a cell surface receptor of a acceptor cell, this receptor is in turn activated, the signal is transduced, modified and amplified in cytoplasm, and in some cases sent to the cell nucleus where it is influences gene transcription. There is a large variety of growth-factors and their corresponding cell-surface receptors participating in this sophisticated communication. Among them are the receptor tyrosine kinases (RTKs), forming the superfamily of 58 proteins encoded in the human genome. This superfamily is divided into 20 families based on structural features, whereby the platelet-derived growth factor receptors are classified in the III family, together with c-Kit, Flt3 and c-Fms.

My thesis adds a small set of data to the ongoing process of understanding PDGF-induced signal transduction, receptor activation, and their down-regulation and biological outputs.

Platelet-derived growth factors and their receptors

Platelet-derived growth factors

Platelet derived growth factor (PDGF), was first purified from platelets in 1970s. It has been characterized as potent growth and migration stimulator of mesenchymal cells [1]. The PDGF family consist of four related polypeptide chains, transcribed and translated from four different genes. PDGF A-

and B-chains considered as the classical PDGFs, are proteolytically cleaved at their N-terminals (both A- and B- isoforms), and at their C-terminals (B-isoform) in order to be activated before secretion to extracellular environment [2]. Moreover, in the C-terminal end PDGF-A and -B have a short sequence which is believed to play a role for their biological properties [3]. Both PDGF-A and -B are closely related in size; 196- 211 amino acid residues and 241 amino acid residues, respectively. PDGF-A and -B can be secreted from donor cells into the intercellular space, but they can also undergo retention on the cell membrane or in extracellular matrix (ECM). This retention is accomplished by a C-terminal amino acid stretch, which is responsible for binding ECM proteins [4]. Recently PDGF-C and –D, were discovered. They are 345 and 370 amino acid residues long, respectively. All four isoforms of PDGF contain a conserved growth factor domain, the PDGF/VEGF homology domain. In addition, PDGF-C and -D have distinct CUB domain in the N-terminals [5, 6]. CUB domain is considered to be involved in protein-protein and protein-carbohydrate interactions and is proteolytically removed from the growth factor domains by extracellular proteases [6]. Importantly, PDGF-C and -D are unable to activate their receptors, unless CUB-domain is removed [5, 6]. PDGF polypeptide chains form both homo- and hetero-dimers, i.e. AA, BB, AB, CC and DD [7]. Formation of PDGF dimers is dependent on the presence of eight highly conserved cysteine residues; two of them participate in formation of the biologically active dimeric ligand bond, while remaining six are involved in forming the structure of each PDGF polypeptide chain [8]. PDGF can bind to extracellular matrix proteins, among them heparan sulphate proteoglycans and collagens, which provide a reservoir of PDGF, important for cell migration, e.g. during process of wound healing [9, 10].

Platelet-derived growth factor receptors

In order to exert a biological function PDGF has to bind and activate its receptor. There are two structurally related receptors able to bind PDGF ligands, i.e. α and β [11, 12]. Both receptors share 30% amino acid sequence identity and domain structures that consists of five extracellular immunoglobulin (Ig)-like domains, a juxtamembrane domain, an intracellular tyrosine kinase domain split by a 100 amino acid residues long stretch, called kinase insert, and a C-terminal tail [13]. This domain structure is common for c-Fms, c-Kit and Flt3 receptors, which together with PDGFRα and β form subclass III of receptor tyrosine kinases [14].

The bivalent ligand brings two monomeric receptors together resulting in receptor dimerization. The second and third Ig-like domains (D2-3) are implicated in ligand binding [15], whereas interaction between critical amino-acid residues of the fourth Ig-like domain (D4), are indispensible for proper platelet-derived growth factor receptor (PDGFR)-β activation, but not for

receptor dimerization [16]. The formation of specific dimeric PDGF receptor complexes depends on which ligands the cell is exposed to, as well as which receptor isoform it expresses [17]. From *in vitro* and *in vivo* studies it is known that PDGF-AA and -CC are able bring together αα-receptors, -DD can form ββ-receptors, -AB, -DD and -CC αβ-heterodimeric receptors, while -BB is able to induce all three type of receptor dimers -αα, -ββ and -αβ **(see Figure 1)**.

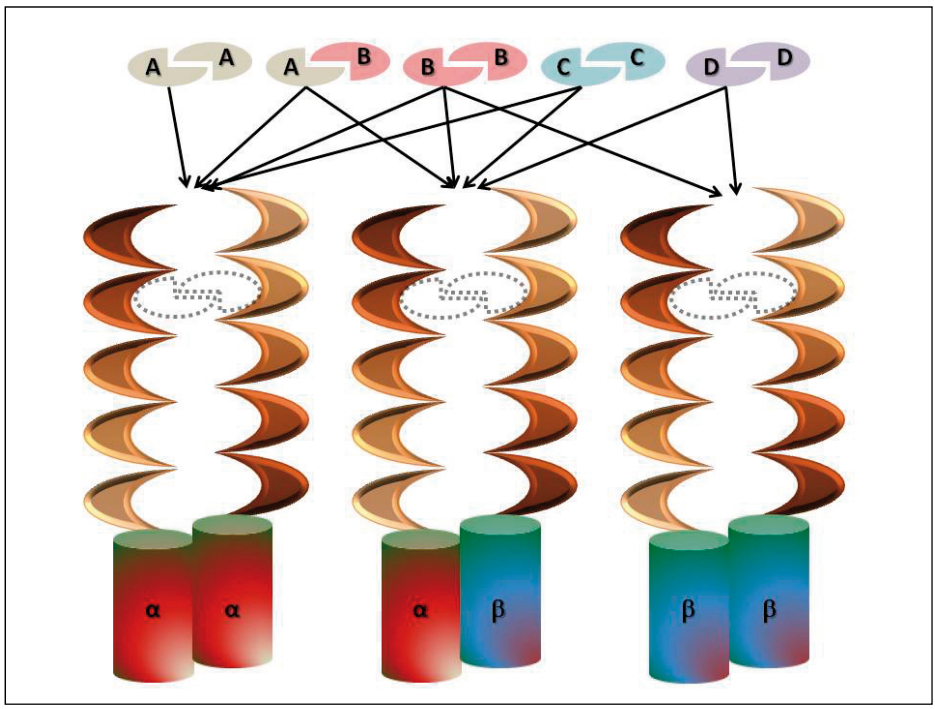


Figure 1. Dimerization variants of PDGF receptors monomers upon binding of different PDGF isoforms.

PDGF receptor activation

The dimerization of receptors is considered as common mechanism of transmembrane receptor activation, including PDGFR [18]. When two monomeric receptors are brought into proximity of each other by ligand binding, conformational changes take place, which leads to an increase in the enzymatic activity of kinase domain resulting in transphosphorylation of certain tyrosine residues located in the intracellular part of the receptors [19, 20]. Simultaneously, the receptor dimer is possibly stabilized by formation of a putative disulfide bonds, upon their transition from intra- to inter-molecule bonds [21]. The strucuture of tyrosine kinases is well conserved among both cytoplasmic and membrane bound RTKs, and consists of two

lobes. The N-terminal lobe is composed of a five β sheets and one α helix and the C-terminal lobe is principally helical in structure. The ATP binds to the phosphate binding loop, located between the two lobes. The phosphate binding loop is a glycine-rich motif (GXGX*Y/F*G). The glycine-rich backbone and aromatic side chains from tyrosine or phenylalanine residues, provides the capacity for phosphate transfer, onto the substrate's tyrosine residue. In order to be phosphorylated a substrate has to reach the active site of the kinase domain, which for most receptors is covered by a 20 to 30 amino acid residues long activation loop. Upon tyrosine phosphorylation of the activation loop of the kinase there is a change in its conformation, opening up the active site of the kinase [22].Most tyrosine kinases has a phosphorylatable tyrosine residue located in the activation loop of receptor kinase domain is well conserved, present in many other membrane receptors, such as IRK [23, 24], fibroblast growth factor receptor (FGFR) [25, 26], brain-derived neurotrophic factor receptor [27] or angiogenic Tie2 receptor [28]. On the contrary, phosphorylation of tyrosine residue located in the activation loop of the epidermal growth factor receptor (EGFR) and c-Kit receptor is not critical for receptor's enzymatic activity [29, 30]. Among the tyrosine autophosphorylation sites for each of the PDGFR isoforms, tyrosine residue 849 (for PDGFR-α), 857 (for PDGFR-β), is located in the activation loop. Phosphorylation of these tyrosine residues is considered of key importance for the enzymatic activity of PDGFRs [31]. Furthermore, also the juxtamembrane domain and C-terminal tail of PDGFRβ are implicated in the regulation of receptor kinase activity. This suggested mechanism of PDGFR kinase activation involves primary phosphorylation of the 579 and 581 tyrosine residues in the juxtamembrane domain, following that the intrinsic kinase transphosphorylates the tyrosine residue 857 in the activation loop, which results in its conformational change which allows for substrates accession [32]. The juxtamembrane domain of PDGFRβ contains a WW domain-like sequence. WW domain recognizes PPXY (proline-proline-X-tyrosine), motifs in proteins. A putative WW domain in the juxtamembrane domain of PDGFRβ may affect phosphorylation of 579/581 tyrosine residues by interacting with unknown PPXY-containing-proteins and thereby regulation of kinase activity [33]. Moreover, studies on other receptor tyrosine kinases, such as EGFR, showed the importance of juxtamembrane domain for the receptor's enzymatic activity. However, EGFR juxtamembrane domain does not require tyrosine phosphorylation, but rather ligand-induced receptor dimerization prompts juxtamembrane domains to promote transmembrane domain rotation to align receptor kinase domains in proper positioning for receptor transphosphorylation [34]. The other intracellular domain of PDGFRβ which is important for regulation of kinase activity is the C-terminal tail. It has been proposed that the C-terminal tail of PDGFRβ changes its conformation upon phosphorylation, to expose the active site of the kinase domain [35]. However, not only tyrosine phosphorylation was shown to play important role in process of PDGFRs kinase activation, but

also cysteine residues 940 and 822 are indispensible for the kinase activity of PDGFRβ. Cysteine residue 940 is involved in the maintenance of receptor structure, while cysteine residue 822 affects receptor's kinase through an unknown mechanism [36]. A second important function of tyrosine autophosphorylation sites is to provide docking sites for PDGFR cytoplasmic substrates containing Src homology2 (SH2) domains and protein tyrosine phosphatase (PTP) domains [37, 38].

Signal transduction

SH2 domain-containing proteins interacting with the PDGFRs

PDGFRα and β have similar but not identical tyrosine phosphorylation patterns **(see Table 1)**.

Table 1. Phosphotyrosine binding signaling proteins interacting with the PDGFRα or β [20, 39].

PDGFRα		**PDGFRβ**	
Tyrosine	*Binding molecule*	*Tyrosine*	*Binding molecule*
572	Src	579	Src/Stat5
574	Src	581	Src/Stat5
720	Shp2	716	Grb2
731	PI3'-K	740	PI3'-K/Shc
742	PI3'-K	751	PI3'-K/Shc/Nck
754	Shp2	763	Shp2
762	Crk	771	RasGAP/Shc
998	PLCγ	775	Grb2/Grb7
1018	PLCγ	857	LMW-PTP
		1009	PLCγ/Shp2
		1021	PLCγ

Binding and downstream signal transduction is correlated with the kinetics of tyrosine autophosphorylation, where some of the tyrosines have more rapid onset or decrease then other [40]. PDGFRs have also been shown to interact with proteins in a phosphorylation independent manner, e.g. Alix that binds to the C-terminal region of PDGFRβ, or NHERF1 which binds to the C-terminal tail of the β-receptor via a PSD-95/Dlg/Z0-1 homology (PDZ)-domain [41, 42].

PDGFRβ-induced signaling pathways

Src family kinases

Src family kinases (SFK) consist of Src, Yes, Fyn, which are ubiquitously expressed, hematopoietic Blk, Fgr, Hck, Lck, Lyn and epithelial Frk. All members of the family share a common domain structure with a SH2, SH3 and tyrosine kinase domain [43]. Src kinase activity is regulated via interaction of its SH2-domain with tyrosine residue 530 located in the C-terminal tail of Src, providing a closed inactive conformation. Phosphorylation of the tyrosine 530 is performed by the C-terminal Src kinase (Csk) and Csk-homology kinase (Chk) [44]. Upon activation of PDGFRβ, the Src SH2-domain binds to phosphorylated tyrosine residues 579 and 581 (579/81) in the juxtamembrane region of the receptor. The release of SH2 domain from phosphorylated tyrosine residue 530 allows Src to adopt an open conformation and undergo activation loop tyrosine 419 phosphorylation [45, 46]. PDGF-induced active Src has been shown to induce *c-myc* mRNA expression and translation [47]. It has also been reported that Src can phosphorylate phospholipase Cγ (PLCγ), which in turn affects Ras guanine nucleotide exchange factor (RasGRP1). Following activation, RasGRP1 translocates to the Golgi apparatus in which it activates Ras, inducing MAP-kinase pathway [48]. Src has been demonstrated to contribute to a mitogenic response, actin cytoskeleton rearrangement and cells motility. Furthermore, Src was shown to phosphorylate tyrosine residue 934 in PDGFRβ, and this phosphorylation decreases the chemotactic response of cells, but increases their mitogenicity [49]. Src is also known to phosphorylate the major PDGFRβ ubiquitin ligase c-Cbl [50]. Phosphorylation of c-Cbl has been reported to regulate its enzymatic function and thereby Src indirectly regulates PDGFRβ downregulation [51].

Erk-MAP-kinase pathway

The Erk-mitogen activated protein (MAP)-kinase pathway is connected to PDGFRβ signaling mostly via Grb2 and Shc. Activated PDGFRβ attracts the Grb2 adaptor protein, through binding of its SH2 domain to tyrosine residue 716 and possibly 775 although this site is phosphorylated at low stoichiometry [52]. Sos is a guanine nucleotide exchange factor that promotes activation of Ras by facilitating the exchange of GDP for GTP. Sos constitutively associates with Grb2. Active Ras activates a cascade of serine/threonine kinase Raf-1, dual specificity kinase map-erk-kinase (Mek) 1/2 and extracellular-regulated kinase (Erk) 1/2 [53]. Furthermore, Shc adaptor proteins can interact simultaneously with both Grb2 and the PDGFRβ forming a bridge between them. Shc has been shown to bind phosphorylated tyrosine residues 579, 740, 751, 771 in PDGFRβ resulting in increased phosphorylation of Shc, which in turn has been connected with mitogenic stimulation [54]. It has also been shown that the activity of Ras is negatively regulated via a GTPase activating protein (GAP), which interacts in SH2-domain dependant

manner to tyrosine residue 771 in PDGFRβ [55]. Activated Erk1/2 phosphorylate and activate several transcription factors, e.g.. Elk1 and c-Fos, which results in stimulation of proliferation, induction of cell differentiation, and possibly enhancement of oncogenic transformation [56]. Erk1/2 is also proposed to stimulate induction of cell cycle control proteins, i.e. induce cyclin D2 and transcription of genes encoding ribosomal proteins and translation factors [57]. In addition Erk1/2 has been shown to enhance mammalian target of rapamacin (mTOR) signaling, which is a kinase involved in integration of growth factor signals to regulate cell growth and cell cycle progression.

Phosphatidylinositol 3'-kinase

Phosphatidylinositol 3'-kinase (PI3'K) is a lipid kinase that is known to bind the PDGFRs and phosphorylate phosphoinositides [58]. PI3'K consists of a p110 catalytic subunit and a regulatory p85 subunit, which contains two SH2 domains that bind to phosphorylated tyrosine residues 740 and 751 in PDGFRβ [59]. Following the binding to active PDGFRβ, the p85 subunit of PI3'K was demonstrated to be phosphorylated, at the tyrosine residue 508 and this may be of importance for PI3'K activity [60]. One of the suggested mechanisms of PI3'K activation involves binding of p85 subunit to PDGFRβ, which subsequently attracts the catalytic subunit p110 to the receptor complex, and the interaction between p85 and p110 possibly results in conformation changes leading to kinase activation of p110. The preferred substrate for PI3'K is one of the cell membrane elements, phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$), being phosphorylated into phosphatidylinositol-3,4,5-triphosphate ($PI(3,4,5)P_3$) [61]. It has been shown that PI3'K activation is not only dependent on PDGFRβ binding, other proteins that are known to interact or to be activated by the PDGFRβ have been implicated as co-activators of PI3'K, i.e. Ras, PLCγ or RasGAP [62]. In the case of Ras it is suggested that GTP-bound Ras interacts with membrane-located PI3'K and thereby provides a proper orientation of PI3'K in reference to its substrate PIP_2. Moreover, PLCγ and RasGAP were shown to have a negative role in controlling PI3'K activity, by competing with PI3'K for the same pools of substrates, i.e. PIP_2 and inactivation of Ras, respectively. Alternatively, PLCγ has been reported to be associated with a serine kinase activity possibly via an interacting protein kinase, and phosphorylation of a serine residue 608 on the p85 subunit of PI3'K was shown to strongly diminish its lipid kinase activity [63]. Activation of PI3'K is involved in cell movement, cell growth and inhibition of apoptosis. A major effector of PI3'K is protein kinase B (PKB) or Akt, a serine/threonine kinase that can bind PIP_3. Interaction between a plekstrin homology (PH) domain of Akt and PIP_3, together with co-localization of phosphoinositide-dependent protein kinase 1 (PDK1), induces conformational changes in Akt, phosphorylation of Akt at threonine residue 308 by PDK1, and phosphorylation of Ser 473 by mTOR, and thereby Akt activation [20, 64]. Downstream effectors of Akt activation are many

proteins involved in growth and survival. In addition, PI3'K activation mobilizes other downstream proteins, e.g. protein kinase C (PKC) family, c-Jun N-terminal kinase (Jnk) and members of the Rho GTP-ase family [53, 65].

Phospholipase C-γ (PLCγ)

Phospholipase C-γ (PLCγ) is activated upon binding to PDGFRβ and phosphorylation on the tyrosine residue 783 [66]. There are two binding sites for PLCγ located in the C-terminal tail of PDGFRβ, i.e. tyrosine residues 1021 and 1009 [67]. The binding occurs via one of two SH2-domains, which together with a SH3-domain, a PH domain and a enzymatic domain comprise the domain structure of PLCγ. Activation of PLCγ results in hydrolysis of PIP_2 to the secondary messengers inositol (1,4,5) triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is responsible for increase in intracellular Ca^{2+} cytoplasmic concentration that in turn activates calcium-dependent proteins. DAG is involved in activation of certain members of the protein kinase C (PKC) family. PLCγ is involved in a wide variety of cellular responses, such as migration, mitogenesis, cell differentiation and transformation, through its ability to activate PKC, increase Ca^{2+} levels and cross-talk with other pathways, i.e. PI3'K, MAP-kinase and Src [20].

Signal transducers and activators of transcription

Signal transducers and activators of transcription (STAT) are a family of transcription factors that are activated by cytokine and growth factor receptors [20]. There is seven members of the Stat family, i.e. Stat1, 2, 3, 4, 5α, 5β and 6, of which 1,3,5α, 5β and 6 were shown to interact with PDGFRβ [68]. The domain structure of all Stat family members is related and consists of a N-terminal conserved DNA-binding domain, a putative SH3-like domain, a SH2 domain and a C-terminal transactivation domain. Tyrosine phosphorylation leads to SH2 domain-mediated homo- or hetero-dimerization of Stat molecules, which triggers translocation to the nucleus where they exert their function as transcription factors. Moreover, it has been demonstrated that additional serine phosphorylation increase the transcriptional activity, of at least Stat1 and Stat3 [69]. Although the classical Stat activation, downstream of a cytokine receptor, requires the receptor-associated Janus kinases (Jak), the PDGF-induced activation of Stat3 [70], and Stat5 [71, 72], do not depend on the presence of activated Jak or Src family kinases. Furthermore, Stat5 is known to directly interact with phosphorylated tyrosine residues 579, 581 and 775 in PDGFRβ and this SH2-domain driven interaction is indispensible for PDGF-induced Stat5 activation [71]. Activated Stats are important for cellular proliferation and cell cycle progression [73].

Fer

Fer, together with Fps/Fes, form a family of the non-receptor protein-tyrosine-kinase (PTK). The overall domain structure is comprised of an N-terminal Fps/Fes/Fer/CIP4 homology (FCH)-domain, followed by three coiled-coils regions, a centrally located SH2 domain and a C-terminal tyrosine kinase domain (**see Figure 2**) [74].

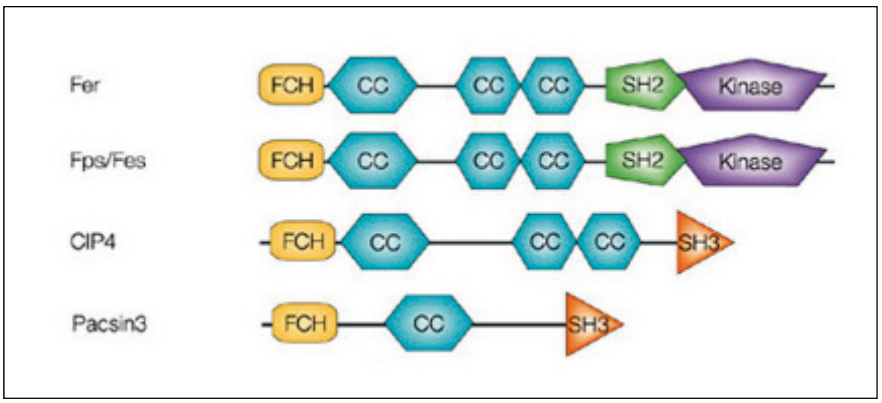


Figure 2. Schematic domain representation of Fps/Fes, Fer, and two Fps/Fes/Fer/CIP4 homology (FCH)-domain family members. The picture has been taken with permission from Greer et al. [74].

The FCH-domain of the structurally related protein CIP4 has been shown to interact with microtubules, while a similar interaction of the Fer FCH-domain has not been observed. However, Fer was still reported to localize to microtubules and play a role in phosphorylation of a platelet endothelial cell adhesion molecule-1 (PECAM-1), in endothelial cells [75]. Further involvement in cytoskeleton organization was demonstrated in the study describing an interaction between Fer and p120 catenin. This interaction facilitates cadherin-β-catenin binding, which allows proper polarization of neurons [76]. The coil-coil regions in Fps/Fes and Fer proteins have been shown to mediate oligomerization, which is a distinct feature of this kinase family [77]. Fer has been shown to form homotrimers, while Fps/Fes can form pentamer and possibly higher-order oligomers [78, 79]. Although oligomerization enhances *trans*-phosphorylation, it does not seem to be necessary for Fer kinase activity [78]. However, deletion of first or second coiled-coil domain results in constitutive activation of the Fps/Fes kinase domain suggesting a role for coil-coil in kinase regulation [80, 81]. Furthermore, the Fer-related testis-specific FerT, naturally lacking coiled-coil-domains, cannot oligomerize and exists as a fully active monomer [78]. The SH2 domains of members of the Fps/Fes/Fer kinase family have been reported to interact with various proteins involved in many cellular functions, i.e. p120 catenin,

EGF and PDGF receptors, p85 subunit of PI3K and insulin receptor substrate-1 (IRS-1) [77, 82]. Both Fer and Fps/Fes have been implicated in many different signaling pathways and been shown to directly phosphorylate and activate several target proteins such as cortactin, p120 catenin, β-catenin, Stat3, Stat5 and SHP-2 [74, 77, 83, 84].

Fps/Fes has been implicated in IL-4 proliferative signaling, located downstream of Jak1/Jak3 [85]. Fps/Fes has been shown to localize at the trans-Golgi apparatus and to exert its kinase activity towards tubulin, which may suggest a role of Fps/Fes in endocytosis, by mobilizing cytoskeleton elements and trans-Golgi for endosomes sorting [86, 87]. Fer has also been shown to be expressed and kinase active in neurons, where it plays a role in Sema3A signaling to microtubular cytoskeleton [87]. Both Fps/Fes and Fer have been show to be activated downstream of collagen receptor glycoprotein VI (GPVI), and lack of these tyrosine kinases results in deregulation of platelet aggregation [88]. Fer has been implicated in regulation of cell-cell contacts, possibly by phosphorylating and activating PTP1B, which in turn interacts with cadherin and dephosphorylates β-catenin. Dephosphorylated β-catenin cannot fully interact with cadherin [89]. Similarly, Fer strengthens cell adhesion by phosphorylating cortactin [90]. Fps/Fes was first identified in myeloid cell lines and has been shown there to take part in hematopoietic differentiation and protect cells from apoptosis [91-93]. In Fer-deficient cells, it has been shown that in order for proper phosphorylation of p38, the presence of Fer is essential, however not for Erk phosphorylation. Full activation of p38 was required to promote migration of antigen-activated mast cells [94]. A knock-out mice lacking Fes/Fps or Fer develop normally, but display defects in hematopoiesis and have reduced fertility [95]. However, studies of mouse mutants with knock-in of kinase-dead Fer have shown that Fer is essential for cortactin phosphorylation, but otherwise seems to be redundant [83].

Protein tyrosine phosphatases

The signal output from a receptor tyrosine kinase can be modulated or inhibited by dephosphorylation. Low-molecular protein tyrosine phosphatase (LMW-PTP) has been shown to negatively regulate PDGFR signaling, by selective dephosphorylation of tyrosine residue 857 in PDGFRβ [96]. CD45, a transmembrane protein tyrosine phosphatase, has similarly been implicated in dephosphorylation of both tyrosine residues in PDGFRβ and phosphorylated substrates, downstream of active PDGFRβ, i.e. RasGAP, p85 subunit of PI3'K and PLCγ [97]. Another PTP, that binds and becomes activated through phosphorylation by PDGFRβ, is SHP-2. SHP-2 is a ubiquitously expressed protein tyrosine phosphatase that possesses two SH2-domains in its N-terminus. It has been reported to selectively dephosphorylate phosphorylated tyrosine residues 771 and 751, and to lower extent 740. [98].

Apart from a negative role in attenuation of PDGFR signaling, SHP-2 also has a positive role in regulation of PDGF-induced downstream signaling.

As mentioned above, SHP-2 dephosphorylates tyrosine residue 771 in PDGFRβ, the binding site for RasGAP which inhibits Ras-MAPK pathway [98]. Furthermore, phosphorylated SHP-2 provides a binding site for Grb2, thus promoting Ras activation [99]. In addition, SHP-2 enhances PDGFRβ induced cell migration, potentially by regulating FAK activation [99, 100]. In contrast to the pro-signaling role of SHP-2, it has been published that in cells depraved of active SHP-2 the mRNA of PDGFRβ is rapidly degraded, which leads to reduced receptor expression [99, 101, 102]. Recently, T-cell protein-tyrosine phosphatase (TC-PTP) has been implicated in site-selective dephosphorylation of activated PDGFRβ, in particular of tyrosine residues 1021 and 771 [103]. Moreover, removal of TC-PTP induces PDGFRβ recycling and thereby decreased receptor degradation. These observations suggest that phosphatases may actively participate in process of receptor sorting towards degradation [104].

PDGFRβ downregulation

Proper regulation of receptor signaling requires their degradation to quench signaling. In this respect, the best studied receptor so far is EGFR, which is generally considered as a model for receptor tyrosine kinase downregulation. The activated receptor undergoes internalization, endosomal trafficking followed by lysosomes fusion and degradation [105]. It has been shown that for the EGFR, efficient cell surface clearance involves association between Grb2 and c-Cbl with the receptor. c-Cbl is an E3 ubiquitin ligase that mediates EGFR monoubiquitination, which targets the receptor to clathin-coated pits, followed by internalization [106, 107].

Ubiquitination

Ubiquitin (Ub), is a 76 amino acid long protein which, following a three-step enzymatic reaction, is covalently attached to a target protein via a bond between a C-terminal glycine residue of ubiquitin and the amino-group of a lysine in the substrate protein [105]. Attachment of ubiquitin to a target protein involves multiple steps. First, ubiquitin is activated by an E1-enzyme, ubiquitin-activating protein, which uses ATP to create a bond between the active-site of the E1-enzyme and the C-terminus of ubiquitin. Second, the E1-enzyme transfers the activated ubiquitin to the active-site of an ubiquitin-conjugating E2-enzyme. Finally, an E3 ubiquitin ligase facilitates the transfer of the ubiquitin moiety from the E2-enzyme to a lysine residue on the substrate [108]. There are three classes of ubiquitination modifications, i.e. monoubiquitination (mono-Ub), where a single Ub moiety is attached to the target protein, polyubiquitination (poly-Ub), where chains of Ub moieties are formed on the target protein, and multiubiquitination (multi-Ub), in which single Ub moieties are attached to more than one lysine residue in targeted

protein. Mono-Ub and multi-Ub has been reported to induce endocytosis, nuclear translocation, transcriptional activity or viral budding. Different modes of polyubiquitination has been shown to regulate diverse processes, for example polyubiquitination via lysine residue 48 (K48) results in proteosomal degradation, while polyubiquitination via lysine residue 63 (K63), influence DNA repair, protein trafficking and ribosomal neo-synthesis [109]. The ubiquitination patterns on targeted proteins are translated into functional responses via proteins containing so called ubiquitin-binding-domains (UBDs), for example; coupling of ubiquitin to ER degradation (CUE), double-sided ubiquitin interacting motif (DUIM), GLUE, GRAM-like ubiquitin-binding in EAP45, histone deacetylase 6 (HDAC6), hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) inverted UIM (IUIM), signal-transducing adapter molecule (STAM), TAK1 binding protein, ubiquitin-associated (UBA), ubiquitin-conjugating (UBC), ubiquitin-binding motif (UBM), ubiquitin-binding protein (UBP), ubiquitin-binding ZnF (UBZ), ubiquitin-conjugating enzyme E2 variant (UEV), ubiquitin-interacting motif (UIM) [110].

Casitas B-lineage lymphoma

The Cbl family of E3-ligases consisting of Cbl-b, Cbl-c/Cbl-3 and c-Cbl, was discovered as an oncogene in casitas B-lineage lymphoma (Cbl) [111-113]. Cbl consists of an N-terminal tyrosine kinase binding (TKB) domain, a RING finger domain and a divergent, proline-rich C-terminal region. The TKB domain mediates interaction with tyrosine phosphorylated proteins. The ubiquitin ligase function of Cbl has been shown to require the TKB domain, although this domain is not considered to be the major RTK-binding domain, e.g. the interaction between c-Cbl and EGFR is belived to be mediated by Grb2 which interacts via its SH3 domain with a proline-rich region of c-Cbl [114]. However, the TKB domain of Cbl does play an important role in interactions with other proteins, such as a negative regulator of Cbl activity Spry2 [115, 116]. Spry2 is proposed to negatively regulate c-Cbl by binding to its RING and TKB domains which in turn prevents complex formation between a receptor and c-Cbl, as well as binding of E2 enzyme to the RING domain of c-Cbl [115]. The RING finger domain of Cbl anchors an E2 ubiquitin-conjugating enzyme [117]. The RING finger domain is separated from TKB domain by a highly conserved linker, which contains two tyrosine residues, 368 and 371, which are essential for the Ub-ligase activity of c-Cbl [118]. The phosphorylation of both tyrosine residue 368 and 371 has been proposed to modulate a critical orientation of RING finger and TKB domains [119]. The C-terminus of the Cbl proteins is not well conserved among species. In case of c-Cbl and Cbl-b this region is proline-rich, containing putative SH3-binding motifs, while Cbl-3 has a very short PRD, with no recognized binding partners [120]. It has been shown that the C-terminal region of c-Cbl is important for interaction with Grb2 [114]. In

addition, the SH3 domain of CIN85, an adaptor protein implicated in EGFR endocytosis and sorting, has been shown to interact with a specific proline-arginine motif (PXXXPR) in c-Cbl, and this interaction is essential for EGFR downregulation [121, 122]. Cbl-b and c-Cbl posses a ubiquitin binding domain (UBA), which is dispensable for Ub-ligase activity but has been implicated in c-Cbl dimerization. Furthermore, the UBA domain has only been shown to interact with Ub for Cbl-b [117, 120, 123, 124]. Studies on PDGFRβ have implicated the low-density lipoprotein 1 (LRP1) in regulation of c-Cbl mediated receptor ubiquitination. In murine fibroblasts, which were depleted of LRP1, association of c-Cbl with PDGFRβ was increased while c-Cbl-driven sorting and degradation of PDGFRβ was abrogated [125]. c-Cbl has been recognized as a major Ub-ligase for PDGFRβ, whereby activation of the receptor resulted in tyrosine phosphorylation of c-Cbl, and PDGFRβ mono-ubiquitination [106]. Furthermore it has been demonstrated that c-Cbl interacts with phosphorylated tyrosine residue 1021 in the PDGFRβ, via its TKB domain, and this is essential for degradation of the receptor [126].

Internalization and endosomal sorting towards degradation

It is generally belived that activated RTKs are internalized via clathrin-coated pits and sorted towards endosomes, which in turn combine with multivesicular bodies and subsequent fusion with lysosomes where they are degraded (**see Figure 3**) [127].

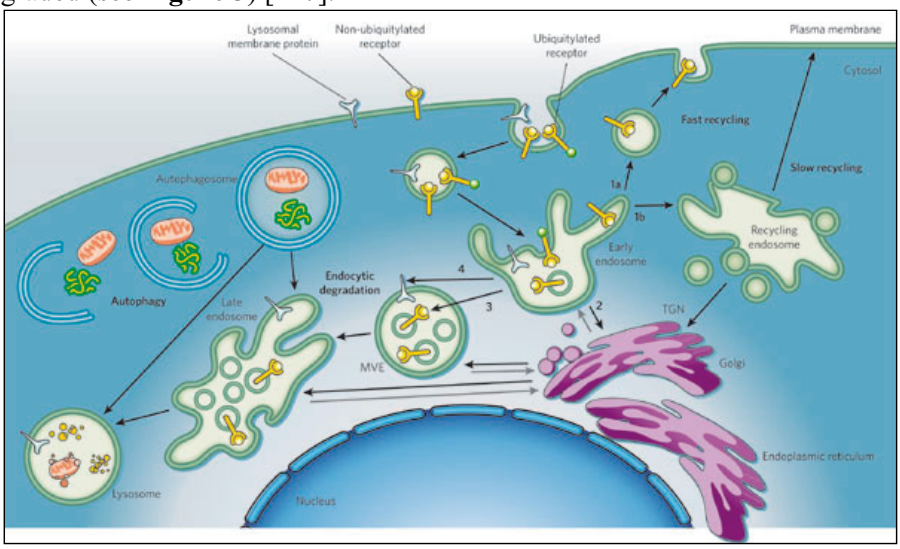
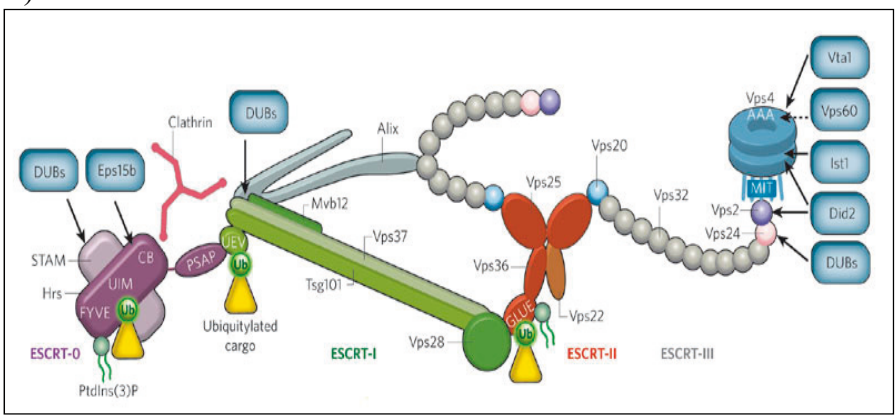


Figure 3. Simplified picture of endocytosis and protein sorting. The nutrient receptors are returned to the cell membrane directly (1a) or indirectly via recycling endosomes (1b). Some membrane receptors, as well as protein toxins, are sorted towards the *trans*-Golgi network (TGN) (2). Growth factor receptors are internalized and sorted via endosomes and multivesicular bodies (MVB) ending up in the lysosomal compartment (3). Lysosomal membrane proteins are sorted to the membranes of MVBs (4). The picture has been taken after permission from Stenmark et al. [128].

Activated PDGFRβ has been shown to be enriched in a membrane structures called caveolae, accompanied by a significant increase in tyrosine phosphorylation of proteins localized in caveolae, e.g. caveolin1 that promotes internalization of molecules in a process of plasma membrane invagination and formation of vesicle-like structures [129]. Moreover, it has been shown that the PDGFRβ kinase activity and the C- terminal tail are required for ubiquitination of the receptor, suggesting that the proline-rich 98 amino acid-long C-terminus is important for efficient PDGFRβ downregulation [130]. The process of EGFR internalization undergoes a dynamic discussion, due to the contradictory data appearing in this field of research. The EGFR downregulation involves ligand-induced dimerization and activation of tyrosine kinase activity, which in turn may attract numerous proteins. It has been shown that the ubiquitin ligase c-Cbl interacts with the EGFR and becomes tyrosine phosphorylated in response to EGF stimulation. This allows anchoring of Cbl-interacting protein of 85 kDa (CIN85), which in turn interacts with endophilin A1. Formation of this complex results in sorting of EGFR towards clathrin-coated compartments in the plasma membrane, followed by formation of clathrin-coated vesicles, under the control from assisting proteins, e.g. Eps15, adaptor protein 2 (AP-2) and dynamin [131-133]. It is generally accepted that the great majority of EGFR is internalized via clathrin-coated pits, however, in cells exposed to extremely high concentrations of EGF, an alternative non-clathrin pathway internalization is available [134]. Interestingly, it has been suggested that although EGFR is ubiquitinated while being present at plasma membrane, this is not obligatory for proper internalization and sorting towards endosomes [135-137]. Furthermore, studies on EGFR internalization showed that it requires presence of PI3P in the vesicle membranes. The PI3P rich endosomal membrane attract hepatocyte growth factor–regulated tyrosine kinase substrate (Hrs), which interacts with PI3P-containing membrane lipids via its FYVE domain, recognizes the ubiquitinated receptor via its UIM domain, and facilitates EGFR sorting towards degradation. Hrs is a compound of the ESCRT-0 complex which will be discussed below [138, 139]. Similar studies on the PDGFRβ internalization and signaling revealed that the receptor is capable of signaling both from cell membrane and from endosomes [140]. Furthermore, there are indications that ligand concentration is of key importance for PDGFR migratory and proliferatory responses. It has been demonstrated that low PDGF concentration can induce migration, but not proliferation, and low rate of receptor degradation, whereas higher concentration of ligand induces cells to proliferate and is sufficient to cause pronounced receptor degradation [141]. This possibly occurs by differential downregulation of active PDGFRβ, which in turn changes output of signaling pathways downstream. This has been observed in studies on EGFR, in which a potent phosphorylation of EGFR, activation of the Erk/MAP-kinase pathway and phosphorylation of the p85 subunit of PI3'K require proper internalization, whereas others such as PLCγ or Shc displayed increased tyrosine phosphorylation in cells depraved of accurate cell surface clearance [142]. EGFR has also been reported to recycle back into the cell membrane. This process occurs either at an early endosome

stage and results in direct sorting back to the membrane, or it passes through distinct type of long-lived endosomes [143, 144]. In order for internalized active receptor to be efficiently transported between the TGN, plasma membrane, MVBs, and lysosomes it needs to be specifically controlled by a protein sorting machinery. In the case of EGFR, this process is composed of formation of endosomes which fuse and create MVBs, this progression occurs in endosomal reticulum membranes [145]. The sorting of MVBs within the TGN membranes requires the multiprotein complex- endosomal sorting complex required for transport (ESCRT), which includes four independent subcomplexes, i.e. ESCRT-0, -1. -2, 3, assisted by ESCRT-associated proteins (**see Figure 4**) [146].

A)



B)

Complex	Yeast protein	Metazoan protein	Mammalian synonym	Ubiquitin-binding domain	Selected interacting proteins (metazoan)
ESCRT-0	Vps27	Hrs	NA	UIM	Clathrin, Eps15b
	Hse1	STAM1, 2	NA	UIM (VHS)	AMSH, UBPY
ESCRT-I	Vps23	Tsg101	NA	UEV	NA
	Vps28	Vps28	NA	NA	NA
	Vps37	Vps37A, B, C, D	NA	NA	NA
	Mvb12	Mvb12A, B	NA	NA	NA
ESCRT-II	Vps22	Vps22	EAP30	NA	NA
	Vps25	Vps25	EAP20	NA	NA
	Vps36	Vps36	EAP45	GLUE	NA
ESCRT-III	Vps2	Vps2A,B	CHMP2A,B	NA	NA
	Vps20	Vps20	CHMP6	NA	NA
	Vps24	Vps24	CHMP3	NA	AMSH, UBPY
	Vps32 (Snf7)	Vps32A, B, C	CHMP4A,B,C	NA	NA
Vps4	Vps4	Vps4A, B	SKD1A,B	NA	NA
	Ist1	Ist1	NA	NA	NA
	Did2 (Vps46)	Vps46A, B	CHMP1A,B	NA	NA
	Vta1	Vta1	LIP5	NA	NA
	Vps60 (Mos10)	Vps60	CHMP5	NA	NA
Other	Bro1 (Vps31)	Alix, HD-PTP	NA	NA	AMSH, UBPY

Figure 4. A) Schematic representation of ESCRT-I,-II,-III complexes. Proteins involved in assembly of core-ESCRTs are assisted by ESCRT-interacting proteins, e.g. Alix and assisting molecules, e.g. PIP3, deubiquitinating enzymes, i.e. Doa4 and ATPase Vps4. B) List of ESCRT- and ESCRT-assisting proteins. The picture has been taken with permission from Stenmark et al. [128].

This conserved protein machinery is responsible for three major functions: firstly to target Ub-modified protein cargoes into endosomes and prevent their recycling or missorting within the cytoplasm, secondly to impose the proper membrane curvature in order to form MVBs, and lastly sort targeted proteins into intralumenal vesicles (ILVs) of MVBs [109]. ESCRT-0 is critical for the EGFR degradation, and is composed of Hrs and Stam proteins assisted by Eps15, which together are important for initiation of the EGFR transportation into the other ESCRT complexes [147]. The ubiquitinated EGFR, bound to ESCRT-0 is then sorted towards ESCRT-I which is a heterotetramer of Tsg101, known to interact with Hrs, vesicle protein sorting (Vps)28, Vps37, and Mvb12 [148]. ESCRT-II consists of Vps36, Vps22 and two Vps25 subunits. Studies on interaction between ESCRT subcomplexes showed that ESCRT-II binds to the Vps28 subunit of ESCRT-I [128]. Following that, ESCRT-II complex transiently interacts with endosomes where it interacts with the ESCRT-III complex [149]. In yeast, the ESCRT-III consists of Vps2, Vps24, Snf7, Vps20 and two additional ESCRT-III-like proteins. i.e. Did2 and Vps60. The binding of ESCRT-III complex to the endosomal membrane is facilitated by myristoyl groups, which are covalently attached to the Vps20 subunits. ESCRT-III then sorts the ubiquitinated cargo protein into the MVB and further sorting towards lysosomes [150]. Finally, ESCRT-III recruits a AAA-type ATPase, Vps4, which induces disassembly of ESCRT-III [128].

Deubiquitination

Before the receptor or other cargo protein enters into MVB they are deubiquitinated. There are 84 active deubiquitinating enzymes (DUBs) in the human genome. The DUBs are necessary for maintaining the ubiquitin pool and it has been shown that there are few DUBs which play central role in receptors sorting, i.e. AMSH and ubiquitin-specific protease Y (UBPY) [151]. STAM, another member of ESCRT-0 subcomplex, has also been shown to interact with two DUBs involved in mammalian RTKs sorting, i.e. UBPY and associated molecule with the Src homology 3 (SH3) domain of STAM (AMSH). The interaction between STAM and AMSH plays a role in the enzymatic activation of AMSH [152]. By knocking-down UBPY and AMSH by siRNA, it has been shown that these proteins may oppose each other in regulation of the EGFR ubiquitination, during the endosomal sorting process; UBPY depletion enhances receptor's accumulation, whereas AMSH depletion induces its more potent degradation [153]. In addition, UBPY has been shown to associate via its SH3 domain with Hrs binding protein (Hbp) [154]. Since Hbp interacts with the ESCRT-0 component Hrs, this will link UBPY to the sorting machinery [155]. Indeed, a study using a deletion mutant of Hbp, which lacks SH3 domain, has reported that this protein variant acts as a dominant negative for PDGFR degradation, suppressing the degradation of PDGF and its receptor, but not the internalization of PDGFR [155].

In yeast cells, proper receptor deubiquitination requires Bro1 (the mammalian homologue is Alix), which is responsible for the recruitment of degradation of alpha 4 (Doa4), a yeast DUB enzyme to the ESCRT-III complex [156].

ALG-2-interacting protein-X (Alix)

Alix, also known as (AIP1), was identified as a binding partner of the ALG-2, a calcium binding protein implicated in apoptosis [157]. It has been shown that Alix plays a role in HIV-1 viral budding by interacting with Tsg101 of ESCRT-1 and with the ESCRT-III component CHMP4 [158-160]. Furthermore, it has been suggested that Alix, together with other molecules, supposedly ESCRTs, controls invagination of lyso-bisphosphatidic acid (LBPA)-containing endosomes in vivo, during the process of membrane fission [161]. Alix domain structure consist of the N-terminal Bro1 domain which mediates localization to endosomes, the C-terminal proline rich domain (PRD), and the inner linker region consisting of two coiled-coil domains (**see Figure 5**) [162].

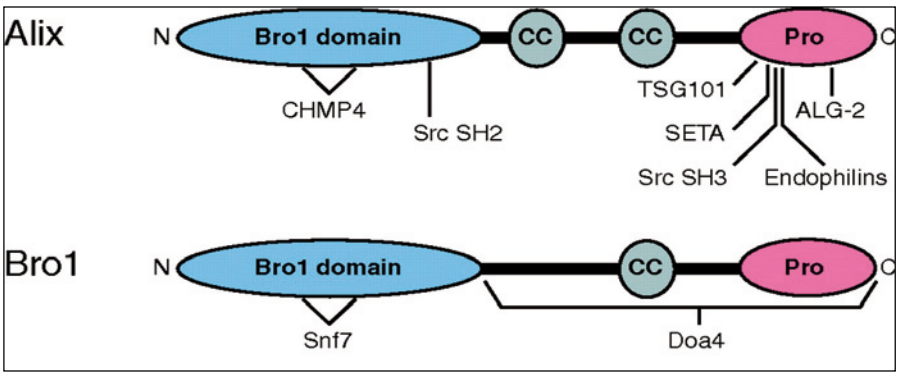


Figure 5. Schematic representation of the domain structure of Alix and its yeast homologue Bro1, with depicted known binding partners. The picture has been taken with permission from Odorizzi et al. [162].

It has been reported that the EGFR sorting into MVBs is controlled by the contradictory functions of Alix and Tsg101 and that overexpression of Alix inhibits intralumenal vesicles (ILVs) formation, while downregulation of Alix resulted in an increase of ILVs formation, possibly through the ability of Alix to affect the fission and fusion processes in MVB formation [163]. In studies on EGFR, Alix has been reported to bind to the receptor in a constitutive manner and this interaction was probably indirect since it could not be observed with recombinant proteins *in vitro* [164]. Moreover, the same study proposed that Alix is a negative regulator of both EGFR internalization

and Cbl-driven EGFR ubiquitination, by interacting with and antagonizing proteins involved in clathrin-coated vesicles formation, i.e. endophilin and CIN85 [131]. In cells depleted of Alix there was an enhanced EGFR internalization, but degradation was normal. This correlated with abnormal, perinuclear localization of early endosomes, whereas late endosomes remained intact and EGFR sorting towards lysosomes was not affected [165]. Furthermore, overexpression of Alix or Alix-CT, a truncation mutant lacking the Bro1 domain, did affect neither dextran- nor clathrin-mediated endocytosis of transferrin. This reported perinuclear localization of endosomes may be related to deformation of intracellular membranes by Alix acting on endophilins, rather not at the plasma membrane but in cytoplasmic compartments [166]. Moreover, Alix interacts with the cytoskeleton and this has been connected to its function in the endocytic route, via an actin-dependent intracellular localization of endosomes, however, this function is not critical for proper lysosomal sorting of downregulated receptors [165]. Alix may provide an alternative bond between ESCRT-I and -III which circumvents the demand for the ESCRT-II in the multicomplex sorting machinery. Therefore, depletion of both Alix and ESCRT-II may be required to inhibit MVB sorting and thus degradation of EGF [153]. However, studies on EGFR sorting, in cells with both ESCRT-II and Alix downregulated, proved this assumption wrong; Alix and ESCRT-II are dispensable for accurate EGFR degradation. This suggests the existence of another interactor(s) that are able to adequately promote EGFR sorting from cell membrane, via endosomes towards the lysosmes [153]. The function and regulation of Alix has not been clarified so far. However, it has been proposed that activated Src can bind to Alix at phosphorylated tyrosine residue 319 and phosphorylate Alix in the C-terminal part. This phosphorylation resulted in Alix departing from the cell membrane and detaching from cytoskeleton, and, in addition, in a reduction in the interaction between Alix and its known binding partners, i.e. SETA/CIN85, EGFR, and Pyk2 [167]. Based on these findings, it may be proposed that a consequence of Src-induced Alix tyrosine-phosphorylation is to oppose the role of Alix as a negative regulator of receptor endocytosis. However, in Bro1 yeast Alix homologue, point mutation of the corresponding tyrosine residue does not affect the MVB sorting [168]. As may be expected from the ability of Alix to interact with the actin cytoskeleton, it has been connected to cell adhesion and cell shape changes. Overexpression of Alix favors cell flattening and spreading, while downregulation of Alix expression promotes opposite effects [169]. Alix has also been shown to directly interact with actin which is required for proper regulation of actin polarization as well as localizing cortactin to the cell periphery [170]. Another function of Alix, discovered before findings implicated Alix in RTKs sorting, is its role in apoptosis. It has been revealed that an overexpression of Alix is able to induce apoptosis in neuronal cell and that this effect comes from changes in Alix interaction with ALG-2. Moreover, some studies showed that loss of the interaction between Alix and ALG-2 or CHMP4b

(member of ESCRT-III subcomplex), but not Tsg101 or endophilin, may rescue cells from apoptosis. Thus, Alix may connect the process of ALG-2 induced apoptosis and RTKs endocytosis [171].

His-domain-containing protein tyrosine phosphatase

The His-domain-containing protein tyrosine phosphatase (HD-PTP) gene was identified in a putative tumor suppressor region of chromosome 3 of the human genome. HD-PTP is considered to be a putative protein tyrosine phosphatase due to a amino acid sequence difference in the active site. A typical PTP displays VHCSAG sequence, whereas HD-PTP has a unique VHCSSG sequence. It is now clear that HD-PTP lacks significant enzymatic activity [172]. Furthermore, back mutation of the amino acid in the active site of HD-PTP that differ from the consensus PTP sequence gave rise to a fully active phosphatase [172] (**see Figure 6**).

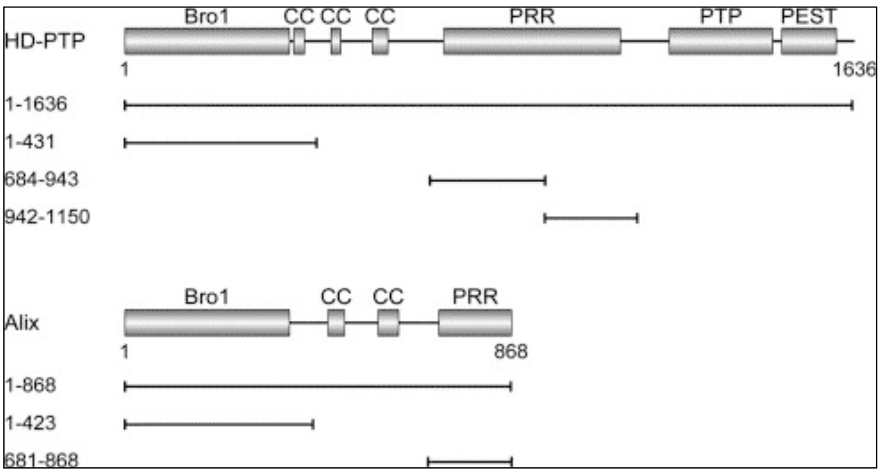


Figure 6. Schematic domain structure of HD-PTP in comparison with Alix. The picture has been taken with permission from Maki et al. [173].

The HD-PTP domain structure resembles that of Alix and contains a N-terminal Bro1 domain, a central His-domain which consist of 14 His and 2 Cys residues and many proline-rich sequences that provide seven putative SH3-domain binding motifs, a putative PTP domain and a C-terminal PEST motif, which is considered to be a destabilization signal [174]. It has been shown that HD-PTP is highly conserved throughout evolution, suggesting its important role in cellular processes. Tat activity, which is an activator of viral genes, has been reported to upregulate PTPs, and cause inhibition of the Erk/MAPK. This has been also observed in case of HD-PTP suggesting its potential role in regulation of virus-related processes. However, while HD-

PTP mRNA level has been shown to be increased by Tat induction, its protein level remained unchanged [175]. Furthermore, PTP-TD14, the rat ortholog of HD-PTP, has been described as a tumor suppressor in Ras-mediated NIH3T3 transformation and this effect required the putative PTP and PEST domains [176, 177]. Previous studies have shown that certain growth factors may induce downregulation of HD-PTP, e.g. FGF2, while others cannot, e.g. VEGF. This downregulation is exerted via proteasomes, which makes HD-PTP to be the first PEST motif-containing PTP regulated via proteosomal degradation [178]. HD-PTP has been also compared with Alix, since they share a large part of their domain structure, and in addition have several common binding partners, such as members of ESCRT-complexes and ESCRT-associated proteins, i.e. Snf7 (CHMP4b), endophilin A1, Tsg101 but not CIN85 [173]. On the other hand, while Alix interacts with the Rab5 GTPase, RabGAPLP, HD-PTP does not, suggesting that Alix rather than HD-PTP play a role in enodosomal trafficking [173, 179].

HD-PTP has also been implicated in cell migration, where downregulation of HD-PTP increased the occurrence of lamellipodia and actin polymerization, suggesting that HD-PTP affects the chemotactic responses of cells [180, 181]. In the same study, HD-PTP has been shown to interact with focal adhesion kinase (FAK), and this binding was dependent on FGF-stimulation, resulting in their detachment upon FGFR activation. Moreover, overexpression of HD-PTP correlated with a significant decrease in FAK phosphorylation, while HD-PTP siRNA silencing resulted in the opposite. Furthermore, long term stimulation of cells with FGF resulted in downregulation of HD-PTP [180, 182]. In line with the proposed role of HD-PTP as the negative regulator of cell migration, in a study concerning EGF-induced migration of T24 human bladder carcinoma cells it has been demonstrated that active EGFR induces tyrosine phosphorylation of HD-PTP and binding to Src and translocation of both proteins to the cell membrane, which in turn increased cell motility [182].

HD-PTP also has a role in intracellular protein sorting, since the absence of HD-PTP reduced EGFR localization to lysosomes and lead to accumulation of ubiquitinated receptors in endosomal compartments. Lack of HD-PTP also perturbed the composition of MVBs, probably due to the critical importance of the Bro1 domain and the central part of protein, which has been shown to form a V-shaped structure, since these domains together were able to rescue EGFR degradation [183]. Mop, the *Drosophila* HD-PTP homologue, has been shown to be important EGFR-dependent processes in the eye and embryonic development of *Drosophila* [184]. In cells lacking Mop, both shape and size of endosomes, as well as EGFR sorting and targeting to lysosomes were impaired. In the same study, the presence of Mops was required for full EGFR induced Erk/MAPK phosphorylation. Moreover, in contrast to HD-PTP, Mop has been shown to act upstream of Ras [184]. Studies in animal models have shown that HD-PTP is an important player in early stages of embryonic development, gastrulation and organogenesis.

Moreover, it has been shown that HD-PTP is highly abundant in epithelia and its absence may cause defects in vascular development [177]. A recent study on potential similarities between HD-PTP and Alix, have shown that both HD-PTP and Alix Bro1 domain displays similar binding capacity towards viral proteins [185, 186]. In cells depleted of HD-PTP it has been demonstrated that EGFR is not sorted properly, lack of HD-PTP resulted in the accumulation of ubiquitinated receptors in endosomes and disordered formation of MVBs [183].

Lysosomal degradation

The lysosome is the final destination for protein sorted via the ESCRT-MVB pathway. Lysosomes are membrane bound vesicles that contain acid hydrolases. Their major function is to digest proteins, which comes from processes of autophagy, endocytosis or another cargo delivery route. There are several possibilities explaining the processing of MVBs into the lysosomes. One of them proposes that endosomes maturate into lysosomes. Alternatively, cargo may be passed from endosomes into lysosomes; a “kiss-and-run” concept has been created, in which there is a constant cycle of short contacts between endosomes and lysosomes during which the transported material is being transferred over into lysosomes. Additionally, there is a model proposing a direct fusion between endosomes and lysosomes creating a hybrid organelle. It is then possible that subsequent fission of the hybrid organelles gives rise to lysosomes.

Present investigation

The biological outcomes of PDGFRβ signaling depend on many aspects, such as receptor autophosphorylation, interaction and activation of directly interacting substrates and subsequent triggering of downstream signaling pathways. In addition, proper intracellular sorting and signaling termination by lysosomal degradation of PDGFRβ provides necessary control. My work explored PDGFRβ activation, downregulation and intracellular signaling pathways. My aims were:

1. To investigate a potential involvement of Alix in PDGFRβ downregulation.
2. To explore whether HD-PTP has a function in sorting and degradation of PDGFRβ.
3. To elucidate the importance of tyrosine residue 857 in the activation loop for PDGFRβ activation and signaling.
4. To study the role of the intracellular kinase Fer in PDGF-induced signal transduction.

Paper I

Alix facilitates the interaction between c-Cbl and platelet-derived growth factor beta-receptor and thereby modulates receptor down-regulation.
Johan Lennartsson, Piotr Wardega, Ulla Engström, Ulf Hellman, Carl-Henrik Heldin.
J Biol Chem. 2006 Dec 22;281(51):39152-8.

In this study we have examined role of Alix (ALG-2 interacting protein X) in regulation of PDGFRβ downregulation. Previous studies has shown that PDGFRβ recruits many binding partners, among them c-Cbl. c-Cbl ubiquitinates PDGFRβ causing its endocytosis and subsequent lysosomal degradation. Since Alix yeast homologue (Bro1) has been shown to anchor ESCRT-(endosomal sorting complex required for transport) I and ESCRT-III, which are crucial for proper sorting of the cell membrane receptors, we wanted to investigate the potential importance of Alix for PDGFRβ downregulation. We found that in porcine aortic endothelial (PAE) cells, Alix constitutively binds to the C-terminal tail of PDGFRβ and becomes tyrosine phosphory-

lated upon PDGF stimulation. In addition, Alix constitutively bound to c-Cbl. We established a population of cells overexpressing Alix and using these we could show that an elevated level of Alix enhanced the interaction between PDGFRβ and c-Cbl. Moreover, under these conditions, c-Cbl became tyrosine hyperphosphorylated and degraded, which correlated with reduced PDGFRβ ubiquitination and reduced cell-surface clearance upon PDGF stimulation.

This study has pointed out the importance of Alix in the downregulation of activated PDGFRβ. By affecting complex formation between PDGFRβ and the ubiquitin ligase c-Cbl, Alix may influence receptor downregulation and therefore affect the spatiotemporal characteristics of PDGFRβ signaling.

Paper II

HD-PTP is important for platelet-derived growth factor beta- receptor downregulation and mitogenicity.
Piotr Wardega, Aleksandra Jurek, Ulla Engström, Carl-Henrik Heldin, Johan Lennartsson
Manuscript

Proper endocytosis and trafficking of activated PDGFRβ is of key importance for the accurate downstream signaling by the receptor. The receptor downregulation process involves cell-surface clearance, fusion of endocytic vesicles with early endosomes, followed by formation of multivesicular bodies (MVBs) and lysosomal degradation. It has been previously shown, that the ubiquitination of activated cell- surface receptors regulates their downregulation processes. HD-PTP (His-domain containing protein tyrosine phosphatase) shares not only most of the domain structure with Alix, but also several binding partners, such as Tsg101 (member of ESCRT-I) or CHMP4 (ESCRT-III). In addition, HD-PTP contains a putative phosphatase domain. The aim of this study was to investigate a possible involvement of HD-PTP in PDGFRβ downregulation. This study was performed in mouse NIH 3T3 fibroblasts and Hek 293T cells. Our data has shown that siRNA-mediated silencing of HD-PTP expression decreased c-Cbl tyrosine phosphorylation upon PDGFRβ activation, but not the interaction between c-Cbl and the receptor. We have shown that HD-PTP is needed for normal PDGFRβ ubiquitination. Interestingly, we observed a decreased rate of PDGFRβ degradation, but no changes in cell-surface clearance, following PDGF-BB stimulation. Furthermore, we have found that the downregulation of HD-PTP resulted in a decreased Erk1/2 phosphorylation, delay in the onset of Akt phosphorylation and completely abolished phosphorylation of p38. Moreover, after treatment of cells with HD-PTP siRNA, we have noted an increased proliferative response following PDGF-BB stimulation. This may be

explained by prolonged presence of activated PDGFRβ in cytoplasm, due to slower degradation, which in turn enables the receptors to signal for longer time in HD-PTP depleted cells, compared to control cells.

These findings suggest an important role of HD-PTP in regulation of PDGFRβ signaling by controlling receptor degradation and thereby its signaling capabilities.

Paper III

Mutation of tyrosine residue 857 in the PDGF β-receptor affects cell proliferation but not migration
Piotr Wardega, Carl-Henrik Heldin, Johan Lennartsson

It has been proposed that the phosphorylation of certain tyrosine residues located in the juxtamembrane domain, activation loop and C-terminal tail of PDGFRβ are important for activation of the kinase domain. In this study we have investigated changes in the PDGFRβ kinase activity, signaling capacity and biological responses, resulting from mutating tyrosine residue 857 to phenylalanine (Y857F). Porcine aortic endothelial (PAE) cells were stably transfected with PDGFRβ wild-type or Y857F or K634R (lysine 634 to arginine point mutant). The K634R mutation abolishes PDGFRβ enzymatic activity, although the receptor may still undergo ligand-driven dimerization. We found that following PDGF-BB stimulation, Y857F PDGFRβ is tyrosine phosphorylated to similar extent as the wild-type receptor, however, the mutant almost completely fails to autophosphorylate in an *in vitro* kinase reaction, as well as to phosphorylate the exogenous substrate myelin basic protein (MBP). We have also shown that some of the PDGFRβ cytoplasmic binding partners are tyrosine phosphorylated to the same extent by Y857F and wild-type PDGFRβ, e.g. c-Cbl, while others are reduced, e.g. SHP-2, Alix and Stam. We have examined the major pathways activated downstream of the PDGFRβ and found that Erk1/2 and Akt phosphorylation was significantly reduced. Interestingly, the rates of Y857F receptor cell- surface clearance and degradation were comparable to wild-type receptor, consistent with the observation that c-Cbl was phosphorylated to the same extent by both mutant and wild-type receptors. The cells expressing Y857F mutated PDGFRβ were less prone to proliferate upon ligand stimulation compared to cells expressing wild-type receptor. This may be associated with the defect in activation of the Erk1/2 and Akt pathways. We found that SHP-2 phosphorylation by Y857F mutant receptor was

strongly reduced. This phosphorylation has previously been reported to correlate with SHP-2 phosphatase activity towards its substrates, e.g. tyrosine residues 771, 751 and 740 in PDGFRβ. Subsequently, we have shown that in cells expressing Y857F mutant receptor phosphorylation of tyrosine residue 771 is indeed increased, which may lead to more pronounced activation of RasGAP, since RasGAP interacts with this particular phosphorylated tyrosine in PDGFRb. Increased RasGAP binding to the receptor can in turn be responsible for lower activity of Ras and subsequent decrease of Erk1/2 activation by Y857F mutant receptor. Alternatively, phosphorylated SHP-2 has been reported to provide docking site for Grb2, a major contributor to the activation of Ras-MAPK pathway.
In contrast, the migratory capacity of cells with the Y857F PDGFRβ mutation in a concentration gradient of PDGF-BB was unaffected. Taken together, our findings showed that mutation of tyrosine residue 857 in PDGFRβ results in negative regulation of two major signaling pathways, Erk1/2 and Akt, downstream of PDGFRβ and selectively attenuate PDGF-driven cell proliferation, while cell migration remains intact.

Paper IV

The Fer tyrosine kinase is necessary for platelet-derived growth factor-BB-induced Stat3 phosphorylation and colony formation in soft agar.
Johan Lennartsson*, Piotr Wardega*, Haisha Ma, Karin Pelka, Ulla Engström, Carina Hellberg and Carl-Henrik Heldin
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*The first two authors contributed equally to this work

Fer is a cytoplasmic tyrosine kinase that has been shown to be activated downstream of the several receptor tyrosine kinases, among them PDGF receptors, but its function in PDGF signaling has remained unclear. Fer has been shown to be activated and phosphorylated upon ligand-induced PDGFR activation. Since Fer was initially identified as a viral oncogene and has been implicated in many cellular processes, such as migration, proliferation and cell adhesion, we investigated its involvement in PDGFRβ signaling and biological responses.

Using NIH3T3 cells and synthetic peptide pull-down approach, we have identified several putative Fer binding sites in PDGFRβ, namely Tyr579, Tyr581, Tyr740 and Tyr1021. We showed that in NIH3T3 cells Fer is probably tyrosine phosphorylated directly by the PDGFRβ, or at least we have excluded Src and Jak family kinases. Moreover, with use of siRNA targeting Fer, we have shown that Fer participates in tyrosine phosphorylation of

PDGFRβ. Our signaling studies revealed that in cells depleted of Fer, the PDGF-induced phosphorylation of Stat3 is abolished. On the contrary, Erk1/2, Akt and Stat5 signaling remained intact. Additionally, we have examined biological responses, such as cell migration, proliferation and colony formation in soft agar. Interestingly, we noted that formation of colonies upon PDGF stimulation was strongly attenuated in cells lacking Fer, while neither proliferation nor migration was affected. The inability of cells to form colonies in soft agar may be related to reduced Stat3 activation in Fer depleted cells since numerous reports have implicated Stat3 in cellular transformation.

Future perspectives

Regulation of receptor signaling is an important task in order to maintain cell homeostasis, and uncontrolled signaling may lead to severe consequences for the organism, for example cancer [187]. Furthermore, there is evidence that receptor mutations may affect RTK activation and their subsequent downregulation [188]. The work in my thesis considers PDGFRβ activity, downstream signal transduction and termination.

We were unable to determine whether the observed lower phosphorylation of SHP-2 affects Erk1/2 phosphorylation by a decreased SHP-2 enzymatic activity, or whether affects the ability of SHP-2 to form a complex with Grb2. The first possibility, that lower phosphorylation of Erk1/2 may be associated with deficient phosphatase activity of SHP-2 can be connected to our hypothesized increased activity of RasGAP on Ras activation, that corresponds with our finding of an increased phosphorylation of tyrosine residue 771, which is both a docking site for RasGAP, as well as a primary dephosphorylation site for SHP-2. The second possibility may be related to the role of SHP-2 as a docking site for Grb2 may thus lead to lowered binding of Grb2/Sos and thus lowered activation of Ras. Experiments with a panel of different SHP-2 variants, i.e. phosphatase-inactive, or SHP-2 without binding site for Grb2 in SHP-2 $^{-/-}$ cells make it possible to draw more definite conclusions on the role of SHP-2 in PDGF-induced Erk1/2 activation. In addition, since we have observed a reduced phosphorylation of Akt kinase, it would be of interest to explore a potential function of SHP-2 upstream of the Akt. We found that the PDGFRβ^{Y857F} could not elicit a mitogenic response whereas the chemotactic response was normal. Further studies are needed to determine whether this is due to a qualitative loss of specific signaling pathways involved in PDGF-induced stimulation of cell growth in the Y857F mutant receptor cells, or whether it is due to a generally lower signaling intensity in these cells.

In order to evaluate the importance of tyrosine residue 857 for its potentially miscellaneous function in PDGFRβ signal transduction regulation it would be of interest to determine the crystal structure of the wild-type and Y857F mutant PDGFRβ. This would not only provide a better view of domain structure of the receptor, but would also help to understand the conformational changes that take place during the receptor kinase activation. Moreover, more work is needed to explain the discrepancy of PDGFRβ autophosphorylation *in vitro* versus *in vivo*. It is possible that an unknown compensating tyrosine kinase exists which can be activated by crippled

Y857F mutant receptor thus allowing this kinase in turn to phosphorylate other tyrosine residues in PDGFRβ. Alternatively, remnant kinase activity in the Y857F mutant receptor is responsible for autophosphorylation *in vitro*, but is for some unclear reason not sufficient *in vivo*.

Having excluded Src and Jak as tyrosine kinases possibly taking part in Y857F mutant receptor phosphorylation, it remains to be elucidated whether Fer can explain the difference between *in vitro* and *in vivo* phosphorylation of the Y857F mutant receptor. siRNA downregulation of Fer lowered the rate of PDGFRβ phosphorylation, suggesting its potential importance for PDGF-induced signaling. Since regulation of PDGFRβ involves autophosphorylation of many tyrosine residues, it will be important to pin-point whether Fer phosphorylates specific tyrosine residue(s) or has a general effect similar to the intrinsic receptor kinase. Additionally, it will be important to explore exactly which downstream effects of PDGFRβ is involving Fer activity. In cells depleted of Fer, PDGF was unable to induce phosphorylation of Stat3, while Stat5, Erk1/2 and Akt remained intact. What is more, we found that cells lacking Fer were unable to form colonies in soft agar. Previous studies have implicated Stat3 activation in cell transformation. Moreover Fer was reported to play a role in cell-cell contact and actin cytoskeleton polymerization. We hypothesize that Fer may promote colony formation by inducing Stat3 phosphorylation and by phosphorylating cell-cell junctions and cytoskeleton proteins. However, the possibility that Fer disables cell-cell contact important for colony formation remains to be more explored in our system, since fibroblast cells do not form epithelial-like cell-cell contacts. Another question is the possible role of Fer in phosphorylation of Stat3. Does Fer directly phosphorylate Stat3? Alternatively, does Fer enzymatic activity has any role in phosphorylation of Stat3? The answer to these questions can be found by transfections of dominant negative variant of Fer. Additionally, of *in vitro* pull-down assays can be used to discover interacting proteins, which can increase our understanding of the role of Fer in colony formation. Moreover, experiments with siRNA targeting Stat3 expression can provide answers whether it is important for colony formation.

Since one of key mechanisms in control of receptor activity is its spatio-temporal sorting and subsequent degradation, we sought to investigate such mechanisms for PDGFRβ. We investigated the adaptor protein molecule Alix and its homologue HD-PTP. We were able to show that siRNA silencing of HD-PTP negatively affects PDGF-induced phosphorylation of c-Cbl. Cells lacking HD-PTP displayed increased PDGF-induced proliferative response. Future experiments should aim to understand the exact mechanism of Alix and HD-PTP in downregulation of PDGFRβ, as well as how Alix or HD-PTP, regulate the interaction between c-Cbl and PDGFRβ is created and regulated. Although previous studies on binding partners and domain structures display many similarities between Alix and HD-PTP, we observed many differences, e.g. both proteins affected c-Cbl phosphorylation, but an interaction could only be observed between Alix and c-Cbl. Use of different

truncation mutants, e.g. lacking Bro1, coil-coil, proline-rich domains, or in the case of HD-PTP, the putative PTP domain, could help to explore how the proteins interact with and which of the domains have roles in PDGFRβ downregulation.

To explore the role of Alix and HD-PTP more broadly it would be of importance to track PDGFRβ from the cell membrane, following PDGF stimulation, to lysosomes, under conditions with reduced Alix or HD-PTP expression. This can be achieved, by analyzing the presence of PDGFRβ and its co-localization with known markers of endosomes (EEA1), recycling endosomes (Rab11) and lysosomes (LAMP-1) using confocal microscopy. With this technique it would be possible to visually dissect the processes of the internalization of PDGFRβ, its subsequent sorting to different classes of endosomes, to analyze the physical characteristics of vesicles containing the receptor, and to explore some of key proteins involved in these intracellular events.

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