NEGATIVE REGULATION OF RECEPTOR TYROSINE KINASES BY T-CELL PROTEIN TYROSINE PHOSPHATASE

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Annales Universitatis Turkuensis, Medica-Odontologica Series D.
Painosalama Oy, Turku, Finland 2009.

ABSTRACT

Protein tyrosine phosphorylation controls a wide array of cellular responses such as growth, migration, proliferation, differentiation, metabolism and cytoskeletal organisation. Tyrosine phosphorylation is a dynamic process involving the competing activities of protein tyrosine kinases and protein tyrosine phosphatases. The protein tyrosine kinases are further divided into non-receptor- and receptor tyrosine kinases. The latter are transmembrane glycoproteins activated by the binding of specific ligands, mostly growth factors, to their extracellular domain, transmitting different signals to the cell. Growth factor receptors such as the epidermal growth factor receptor, vascular endothelial growth factor receptor 2 and platelet-derived growth factor receptor β, belong to the receptor tyrosine kinases, the signalling of which is often disturbed in various diseases, including cancer. This has led to the development of receptor tyrosine kinase antagonists for use as anti-cancer drugs.

As the receptor tyrosine kinases, also the protein tyrosine phosphatases can be divided into receptor- and non-receptor types. The protein tyrosine phosphatases have attained much less attention than the receptor tyrosine kinases partly because they were identified later. However, accumulating evidence shows that the protein tyrosine phosphatases have important roles as specific and active regulators of tyrosine phosphorylation in cells and of physiological processes. Consequently, the protein tyrosine phosphatases are receiving arising interest as novel drug targets.

The aim of this work was to elucidate the negative regulation of receptor tyrosine kinases by one non-receptor protein tyrosine phosphatase, T-cell protein tyrosine phosphatase TCPTP. The results show that TCPTP activated by cell adhesion receptor integrin α1 functions as a negative regulator of the epidermal growth factor receptor. It was also found that TCPTP affects vascular endothelial growth factor receptor 2 signalling and angiogenesis. Lastly, a High-throughput screen with 64,280 compounds was performed to identify novel TCPTP activators, resulting in identification of one small molecule compound capable of exerting similar effects on TCPTP signalling as integrin α1. This compound is shown to downregulate signalling of epidermal growth factor receptor and platelet-derived growth factor receptor β, as well as to inhibit cell proliferation and angiogenesis. Our results suggest that a suitable small-molecule TCPTP activator could be utilized in the development of novel anti-cancer drugs.

Keywords: Receptor tyrosine kinase, Protein tyrosine phosphatase, TCPTP, cancer, growth factor receptor, angiogenesis

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T-solun proteiinityrosiinifosfataasi reseptorityrosiinikinaasien negatiivisena säätelijänä

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TIIVISTELMÄ

Monia solun toimintoja kuten kasvua, liikkumista, jakautumista, erilaistumista, aineen-vaihduntaa ja solurangan järjestäytymistä säädellään fosforyloimalla proteiinien tyrosii-neja. Tyrosiinien fosforylointi on dynaaminen prosessi, jossa vastakkaisina toimijoina ovat proteiinityrosiinikinaasit ja proteiinityrosiinifosfataasit. Proteiinityrosiinikinaasit jaotellaan ei-reseptori-tyrosiinikinaaseihin ja reseptorityrosiinikinaaseihin. Jälkimmäi-set ovat solukalvon läpi ulottuvia glykoproteiineja, jotka aktivoituvat ligandin, usein kasvutekijän, sitoutuessa solunulkoiseen osaan, välittäen viestejä solun sisälle. Resepto-rityrosiinikinaasien perheeseen kuuluvat mm. epidermaalinen kasvutekijä, verisuonten kasvutekijä 2 ja verihiutalekasvutekijä β. Monissa taudeissa, kuten syövässä, reseptori-tyrosiinikinaasien viestintä on häiriintynyt. Tämä on johtanut reseptorityrosiinikinaasi-antagonistien kehitykseen syöpälääkkeiksi.

Myös proteiinityrosiinifosfataasit jaotellaan reseptori- ja ei-reseptorityyppeihin. Pro-teiinityrosiinifosfataasit löydettiin myöhemmin kuin reseptorityrosiinikinaasit, mikä on osaltaan syynä niiden suppeampaan tutkimukseen. Tulokset kuitenkin osoittavat niiden tärkeän roolin tyrosiinifosforylaation spesifisinä ja aktiivisina säätelijöinä sekä fysiolo-gissa prosesseissa. Tällä hetkellä proteiinityrosiinifosfataasit ovat suuren kiinnostuksen kohteena syöpälääkkeiden kehittelyssä.

Tämän väitöskirjatutkimuksen tavoitteena oli selvittää reseptorityrosiinikinaasien negatiivista säätelyä T-solun proteiinityrosiinifosfataasin (TCPTP) toimesta. TCPTP kuuluu ei-reseptori-proteiinityrosiinifosfataasien perheeseen. Tuloksemme osoittavat, että soluadheesioreseptori integriini α1:n aktivoima TCPTP estää epidermaalisen kasvu-tekijäreseptorin toimintaa. Aktivoidun TCPTP:n havaittiin myöskin vaikuttavan nega-tiivisesti verisuonten kasvutekijä 2:n toimintaan sekä verisuonten uudismuodostukseen. Työssä tehtiin lisäksi high-throughput -seulontatutkimus, jossa etsittiin uusia TCPTP-aktivaattoreita 64,280 pienimolekyylisen yhdisteen joukosta. Työn tuloksena löytyi yksi yhdiste, jolla oli α1-integriinin kaltaisia vaikutuksia TCPTP:n signalointiin. Tämän yhdisteen näytettiin pystyvän vähentämään epidermaalisen kasvutekijäreseptorin ja ve-rihiutalekasvutekijäreseptori β:n viestintää, sekä solujen jakaantumista ja verisuonten uudismuodostusta. Tuloksemme osoittavat, että sopivaa pienimolekyylistä TCPTP-akti-vaattoria voitaisiin mahdollisesti hyödyntää uuden syöpälääkkeen kehittelyssä.

Avainsanat: Reseptorityrosiinikinaasi, proteiinityrosiinifosfataasi, TCPTP, syöpä, kas-vutekijäreseptori, verisuonten uudismuodostus

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ABBREVIATIONS

CSF-1	Colony stimulating factor 1
CSF-1R	Colony stimulating factor 1 –receptor
DEP-1	Density-enhanced phosphatase 1
DSP	Dual-specificity phosphatase
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FAK	Focal achesion kinase
FBS	Fetal bovine serum
FERM	Band 4.1, Ezrin, Radixin, Moesin
GEF	Guanine nucleotide exchange factor
GSK3	Glycogen synthase kinase 3
ko	Knockout
HGF	Hepatocyte growth factor
HTS	High-throughput screen
IR	Insulin receptor
JAK	Janus family of tyrosine kinases
JNK	Jun amino-terminal kinase
MEF	Mouse embryonic fibroblast
Met	Hepatocyte growth factor receptor
NLS	Nuclear localisation signal
NPC	Nuclear pore complex
PDGF	Platelet-derived growth factor
PDGFRβ	Platelet-derived growth factor receptor β
PDZ	Postsynaptic density-95 – discs large – zonula occludens 1
PI3K	Phosphatidylinositol 3-kinase
PIP	Phosphoinositide phosphate
PlGF	Placental growth factor
PRD	Proline-recognition domain

PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
nrPTP	Non-receptor-type protein tyrosine phosphatase
rPTP	Receptor-type protein tyrosine phosphatase
PTP1B	Protein tyrosine phosphatase 1B
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SFK	Src-family kinase
SH2	Src homology 2 –domain
SH3	Src homology 3 –domain
SHP1	Src homology 2 –domain containing tyrosine phosphatase 1
SHP2	Src homology 2 –domain containing tyrosine phosphatase 2
SOS	Son of sevenless
STAT	Signal transducers and activators of transcription
S/TP	Serine/threonine phosphatase
TCPTP	T-cell protein tyrosine phosphatase
TCPTP-D182A	Substrate-trapping form of TCPTP
TC45	45 kDa form of TCPTP
TC48	48 kDa form of TCPTP
Tyr	Tyrosine
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
wt	Wild type

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals I-III. Unpublished data is also included.

I   Elina Mattila, Teijo Pellinen, Jonna Nevo, Karoliina Vuoriluoto, Antti Arjonen and Johanna Ivaska. (2005) Negative regulation of EGFR signalling through integrin α1β1-mediated activation of protein tyrosine phosphatase TCPTP. *Nature Cell Biol.* 7(1): 78-85.

II   Elina Mattila, Kaisa Auvinen, Marko Salmi and Johanna Ivaska. (2008) The Protein Tyrosine Phosphatase TCPTP controls VEGFR2 signalling. *J Cell Sci.* 121(21): 3570-80.

III   Elina Mattila, Heidi Marttila, Niko Sahlberg, Pekka Kohonen, Jari Ylänne, Mikko Huhtala, Jarmo Käpylä, Pasi Halonen, Merja Perälä and Johanna Ivaska. Novel protein tyrosine phosphatase TCPTP activator attenuates RTK signalling. *Manuscript*.

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1.   INTRODUCTION

Numerous signalling events in the cell are controlled by the opposing actions of the protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). These events include communication between and within cells, regulation of gene transcription, mRNA processing, proliferation, differentiation, mobility, and transport of molecules in and out of cells. Receptor tyrosine kinases (RTKs), belonging to the group of PTKs along with non-receptor tyrosine kinases, are high-affinity transmembrane receptors that bind many polypeptide growth factors, cytokines and hormones. The PTPs in turn can be subgrouped into classical phosphotyrosine-specific phosphatases and dual-specificity PTPs (DSPs), and the classical PTPs are further divided into non-receptor- and receptor-PTPs (nrPTPs and rPTPs).

Many RTKs have been shown to be regulated by several PTPs. To date, due to intensive research, the largest numbers of PTPs have been identified for insulin receptor (IR) and epidermal growth factor receptor (EGFR) downregulation. Several RTK inhibitors are currently on the market and in clinical trials. However, also PTP-antagonists have emerged as highly interesting candidates for drug design. The first PTP-inhibitors, targeted against PTP1B, are currently in clinical trials for type II diabetes and obesity.

T-cell protein tyrosine phosphatase (TCPTP), a ubiquitously expressed nrPTP, has been shown to downregulate signalling of six RTKs, namely EGFR, vascular endothelial growth factor receptor 2 (VEGFR2), IR, platelet-derived growth factor receptor β (PDGFRβ), Met/Hepatocyte growth factor (HGF) receptor (Met) and colony-stimulating factor 1 –receptor (CSF1-R). The downregulation is site-specific. In addition to the RTKs, TCPTP regulates signalling of the Janus family of tyrosine kinases (JAKs), signal transducers and activators of transcription (STATs), as well as Src-family kinases (SFK) that belong to the non-receptor tyrosine kinases.

In this study, the role of TCPTP in the negative regulation of the RTKs was elucidated. In addition, the activation mechanism of TCPTP by the α1 integrin was studied.

2. REVIEW OF THE LITERATURE

2.1. Receptor tyrosine kinases

2.1.1. Protein tyrosine phosphorylation

Reversible phosphorylation of proteins is an important regulatory mechanism that occurs in both prokaryotes and eukaryotes. Kinases phosphorylate and phosphatases dephosphorylate proteins, causing many enzymes and receptors to be activated or inactivated. In eukaryotic proteins there are three potential amino acid residues at which phosphorylation can occur: serine, threonine, and tyrosine. As their name implies, tyrosine kinases are specific for tyrosine (Tyr) residues whereas other kinases phosphorylate serine, threonine, or both.

Phosphorylation of key proteins on specific tyrosine residues is a fundamental mechanism by which cells control their growth, proliferation and differentiation (Hendriks et al. 2008). Only approximately 0,1 % of the cellular phosphoamino acid content is made up by phosphotyrosines, but their role in cell signalling is disproportionate to that (Stoker 2005). Various stimuli, such as growth factors, hormones, cytokines, extracellular matrix (ECM) components, and cell adhesion molecules transmit signals via pathways that involve tyrosyl phosphorylation of specific cellular proteins. The fate of the cell depends on which pathway is chosen: the cell either grows and divides, moves, changes shape, differentiates or dies (Neel, Tonks 1997). Defects in tyrosine phosphorylation are involved in the pathogenesis of many inherited or acquired human diseases from cancer to immune deficiencies.

Tyrosine phosphorylation is controlled by the coordinated actions of protein tyrosine kinases (PTKs) and phosphatases (PTPs) (Figure 1). When inactive, proteins are non-phosphorylated, and become activated by the phosphorylation. Originally, PTKs were regarded as the key enzymes controlling tyrosine phosphorylation, leaving PTPs with housekeeping functions. Today, PTPs are recognized as being able to selectively dephosphorylate phosphotyrosine residues on their substrates, thus having an important role in initiating, maintaining, and terminating cellular signalling (Alonso et al. 2004).
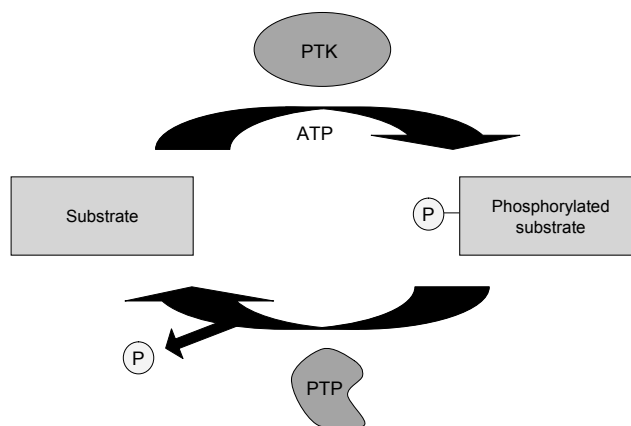
Figure 1. The cycle of phosphorylation. Proteins are phosphorylated on tyrosine residues by the action of protein tyrosine kinases (PTK), and dephosphorylated by protein tyrosine phosphatases (PTP). Adapted from (Mustelin, Vang & Bottini 2005).

2.1.2. Definition and general characteristics

Receptor tyrosine kinases are transmembrane proteins having their ligand-binding domain on the outer surface of the plasma membrane. There are 58 different RTKs, and based on their structural extracellular characteristics they can be divided into 20 structural subfamilies, each having its own specific family of protein ligands (Robinson, Wu & Lin 2000). In Figure 2 are shown the structures of the five TCPTP-regulated RTK subfamilies that are dealt with in this thesis. The RTKs belong to the group of protein tyrosine kinases, together with nonreceptor tyrosine kinases. The ligand-binding domain is connected to the cytoplasmic domain by a single transmembrane helix. The cytosolic domain of RTKs, which either has intrinsic enzyme activity or associates directly with an enzyme, consists of a conserved PTK core and additional regulatory sequences (Brunelleschi et al. 2002). RTKs have extracellular domains such as immunoglobulin-like domains (IG) and fibronectin type III domains (FN), which assist in binding of the cell to other cells and to the ECM.

As the name implies, the RTKs phosphorylate specific tyrosines on intracellular signalling proteins. They transmit signals on differentiation, proliferation, migration and invasion, angiogenesis and survival throughout the cell. The extracellular signal proteins acting via RTKs consist of a wide variety of secreted growth factors such as EGF, VEGF, HGF, PDGF, insulin, and hormones. Also many signal proteins bound to the cell surface act through RTKs. The main feature shared by the members of the RTK family members is the intrinsic enzymatic activity that catalyzes the transfer of the γ-phosphate of ATP to tyrosine residues in protein substrates (Brunelleschi et al. 2002). All the known RTKs

reside as monomers in the cell membrane when inactive. The only exception is presented by the insulin receptor, which is a dimer in its inactive form (Figure 2) and tetramerizes upon activation.
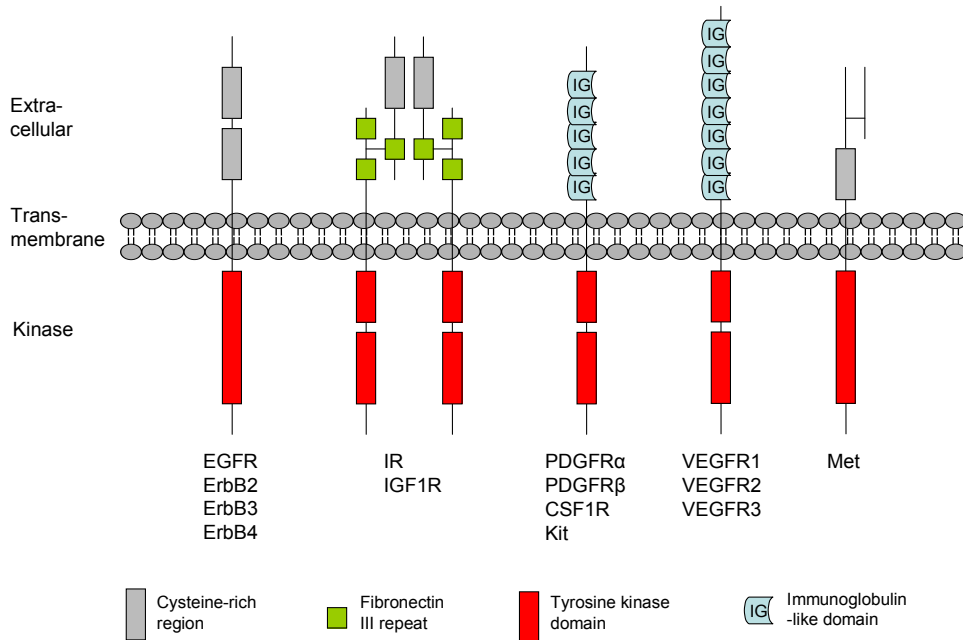
Figure 2. Schematic view of the domain composition of selected RTKs. Adapted from (Hubbard, Till 2000).

Activation of all RTKs proceeds in a similar manner (Figure 3). First the binding of a ligand to the extracellular ligand-binding domain induces dimerization of two RTKs, forming homo- or heterodimers (or –tetramers in the case of IR). Then the intracellular, neighbouring tyrosine kinase domains phosphorylate each other in a process called transphosphorylation where the γ-phosphate from ATP is transferred to selected tyrosine side chains, usually located in the non-catalytic regions of the receptor molecule. After ligand-induced transphosphorylation, each RTK displays a distinct array of phosphotyrosine residues on its cytoplasmic tail. The uniqueness of each of these phosphotyrosines is determined by the sequence of amino acid residues flanking the phosphotyrosine on its C-terminal side. These phosphotyrosines and their adjacent sequences then attract various downstream signalling molecules containing SH2 (Src-homology 2) or PTB (phosphotyrosine binding) domains, and phosphorylate them in turn, promoting the signalling pathway in question (Brunelleschi et al. 2002). Several of the proteins containing SH2 – or PTB –domains possess intrinsic enzymatic activities

(Src kinases, SHP2, PLCγ) and/or protein modules that mediate interactions with other proteins, phospholipids or nucleic acids. Many of these protein modules are involved in cellular signalling downstream of RTKs and other cell surface receptors, thus mediating constitutive or ligand-dependent associations (Heldin 2001).
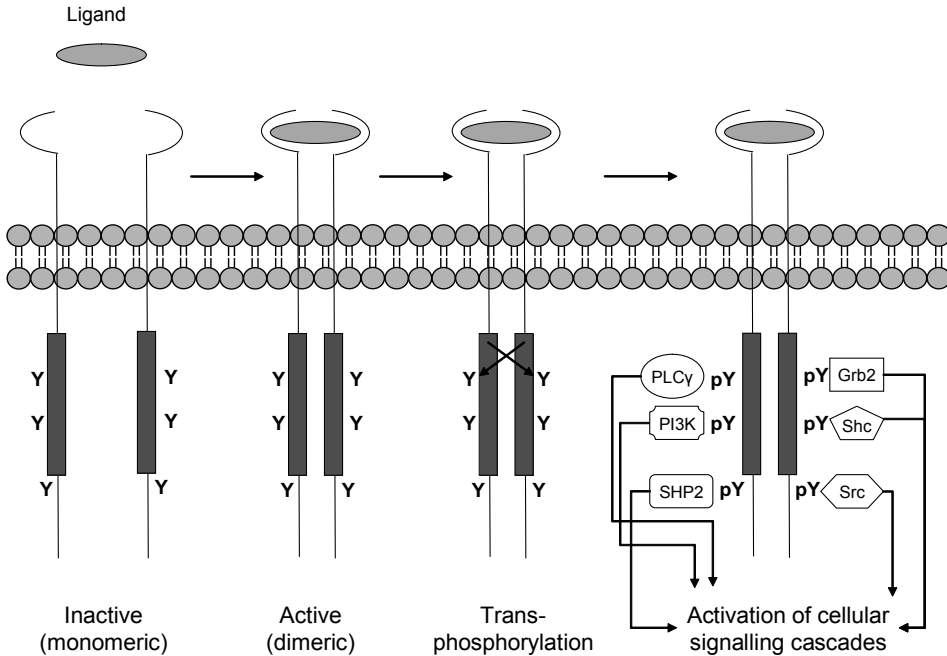
Figure 3. General activation mechanism of the RTKs.

RTKs can also be activated ligand-independently (Ostman, Bohmer 2001), since receptor autophosphorylation can be achieved either by using PTP inhibitors (Jallal, Schlessinger & Ullrich 1992) or by overexpressing the RTK in question (Brunelleschi et al. 2002).

Amplification of ErbB2, a member of the ErbB –family of RTKs together with EGFR, is detected in the tumors of about 25 % of all women diagnosed with early breast cancer, the most common female malignancy in many industrialized countries. The selective monoclonal antibody trastuzumab (Herceptin) is used in the treatment of breast cancer, together with chemotherapy (Singer, Kostler & Hudelist 2008). RTKs can be constitutively activated by a mutation in the extracellular or kinase domain, for example alteration of the activation loop, the ATP binding site or the substrate-binding pocket. The occurrence of constitutively activated receptors is involved in a number of human diseases, e.g. EGFR and Met –receptors in cancer (Joughin et al. 2009, Robertson, Tynan & Donoghue 2000). Gastrointestinal stromal tumors (GISTs), the most common mesenchymal tumors of the gastrointestinal tract, often have activating mutations in

RTK KIT (75-80 %) or PDGFRα (5-10 %). This leads to ligand-independent signal transduction. A small-molecule kinase inhibitor imatinib mesylate (Gleevec) is used to treat recurrent or metastatic GISTs, although 50 % of patients become resistant to Imatinib after approximately two years of treatment (Braconi, Bracci & Cellerino 2008). RTKs can also experience loss-of-function mutations, leading to non-functional or dominant negative receptors (Robertson, Tynan & Donoghue 2000).

Downregulation of RTKs can occur via several mechanisms, including receptor-mediated endocytosis, ubiquitin-directed proteolysis, and the action of the PTPs (Hubbard, Till 2000), which will be discussed later.

RTK signalling is often disturbed in cell transformation and in several diseases. This has led to the development of RTK antagonists for use as anti-cancer drugs or against diabetes mellitus type II (Ostman, Bohmer 2001).

More detailed information is provided below of those six RTKs that have either previously (clarified in 2.4.) or by our results (clarified in the Results and Discussion –sections) been shown to be targets of the T-cell protein tyrosine phosphatase (TCPTP).

2.1.3. Epidermal growth factor receptor

Four transmembrane RTKs belong to the epidermal growth factor receptor (EGFR) family, namely EGFR (ErbB1), Her2 (ErbB2), Her3 (ErbB3) and Her4 (ErbB4). They form homo- and heterodimers following ligand binding, each dimer having different affinity for ligands and different signalling properties (Normanno et al. 2006). Epidermal growth factor receptor EGFR is a 173 kDa RTK that is predominantly located at the basolateral surface of polarised epithelial cells. The mammalian EGFR is bound by several ligands with various affinities: EGF, transforming growth factor α (TGFα), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin, epigen and epiregulin (Normanno et al. 2006).

EGFR has been highly conserved during evolution. It controls a wide range of cellular processes such as proliferation, cell migration, cell fate determination, and apoptosis (Normanno et al. 2006). EGFR has an essential role in signalling pathways controlling the growth of normal and malignant cells (Hynes, Lane 2005). Its activation proceeds in the manner described for the RTKs in general (above), with ligand-induced dimerization and subsequent transphosphorylation of the phosphotyrosines in the intracellular portion of the receptor. In the absence of a ligand, EGFR stays in a conformation that suppresses kinase activity and restrains formation of receptor dimers. Binding of a ligand initiates a conformational alteration that unmasks a "dimerization loop", triggering receptor dimerization (Yarden, Shilo 2007). The EGFR monomer dimerizes either with another EGFR monomer, or with another family member: ErbB2, ErbB3, or ErbB4. Interestingly,

binding affinity of EGF towards EGFR is affected by co-expression of ErbB2 or ErbB3 in the same cells (Singh, Harris 2005). Triggered by EGFR kinase activation, several downstream signalling pathways can be activated, depending on the set of adaptor proteins binding to the phosphotyrosines of EGFR. These pathways include the small GTP-binding protein Ras / extracellular signal regulated kinase (ERK) pathway, the phosphatidylinositol-3-kinase (PI3K) / Akt pathway and the Janus kinase / Signal transducer and activator of transcription (JAK/STAT) pathway. PI3Ks are a family of enzymes phosphorylating phosphoinositides, their effectors including serine/threonine kinases such as Akt/PKB, specific members of the PKC family, and small GTPases of the Rho family.

The ERK pathway is one of the mitogen-activated protein kinases (MAPKs) that mediate signals in cells. It is activated through the binding of SH2-domain containing adaptor proteins Grb2 or Shc to phosphorylated EGFR family receptors, resulting in the recruitment of the guanine nucleotide exchange protein son of sevenless (SOS) to the activated receptor dimer via the SH3-domain of Grb2/Shc. SOS activates Ras, leading to the activation of MAP kinase kinase kinase Raf-1, which in turn phosphorylates MAPK/ERK kinases MEK1 and MEK2 to activate ERK1 and ERK2, respectively. This pathway promotes cell survival, by resulting in cell proliferation and in the increased transcription of Bcl-2 family members and inhibitor of apoptosis proteins (Henson, Gibson 2006).

EGF promotes cell survival also via activation of PI3-kinase / Akt signalling (Henson, Gibson 2006). PI3K binds to specific phosphotyrosines of the EGFR-family receptors via its SH2-domain. The catalytic subunit of PI3K phosphorylates phosphatidylinositol(4,5) bisphosphate (PIP2), leading to the formation of phosphatidylinositol(3,4,5)trisphosphate (PIP3). Alternatively, PI3K can activate Ras, resulting in the activation of ERK signalling. A key downstream effector of PIP3 is Akt/PKB, which promotes cell survival via the transcription of anti-apoptotic proteins, utilizing the transcription factors Nuclear factor-κB (NFκB) and cAMP response element binding CREB. Glycogen synthase kinase 3 (GSK3) is another downstream target of Akt. It is constitutively active in basal conditions, leading to the phosphorylation and inhibition of a guanine nucleotide exchange factor eIF2B, which regulates the initiation of protein translation. Therefore, as a result of inactivation of GSK3 by Akt, eIF2B is dephosphorylated leading to the promotion of protein synthesis and the storage of amino acids (Lizcano, Alessi 2002). Akt also activates mammalian target of rapamycin (mTOR), which promotes protein synthesis through p70 ribosomal S6 kinase (p70s6k) and inhibition of eIF-4E binding protein (4E-BP1) (Asnaghi et al. 2004).

Third signalling pathway initiated by binding of EGF to EGFR is the JAK/STAT pathway, also involved in cell survival responses (Kisseleva et al. 2002). JAK phosphorylates STAT proteins localized at the plasma membrane, leading to the translocation of STATs to the nucleus where they activate the transcription of genes associated with cell survival. To summarize, all these abovementioned pathways promote cell growth and survival in response to EGF.

EGFR is overexpressed in many different tumor types, and majority of human epithelial cancers are characterized by functional activation of growth factors and receptors of the EGFR family (Ciardiello, Tortora 2008). Not surprisingly, EGFR was the first growth factor receptor that was assessed as a target for cancer therapy, and the first anti-EGFR drugs were developed in 1980s (Hynes, Lane 2005, Masui et al. 1984). Two different classes of EGFR antagonists have successfully passed phase 3 trials and are now in clinical use: anti-EGFR monoclonal antibodies (cetuximab and panitumumab) and small-molecule EGFR tyrosine kinase inhibitors (erlotinib and gefitinib) (Ciardiello, Tortora 2008, Ciardiello, Tortora 2001, Normanno et al. 2003). All of the four drugs are at present used in treating four metastatic epithelial cancers: non-small-cell lung cancer (erlotinib, gefitinib), squamous-cell carcinoma of the head and neck (cetuximab), colorectal cancer (cetuximab, panitumumab), and pancreatic cancer (erlotinib) (Ciardiello, Tortora 2008).

2.1.4. Vascular endothelial growth factor receptor 2 and angiogenesis

2.1.4.1. VEGFR2

Vascular endothelial growth factors (VEGFs) are homodimeric glycoproteins that are essential for the development of the blood vascular system of the embryo (vasculogenesis), lymphatic system (lymphangiogenesis), and for the formation of new blood vessels from pre-existing ones (angiogenesis). Deletion of a single allele of the VEGF gene results in early embryonic lethality. Five different VEGF ligands have been identified in mammals, namely VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). The VEGF ligands mediate their responses via three structurally related VEGF-receptor (VEGFR) tyrosine kinases. VEGFR1 (Flt1) mediates recruitment of haematopoietic stem cells as well as the migration of monocytes and macrophages. VEGFR2 (Flk-1/ KDR) is essential in physiological and pathological angiogenesis through specific signalling pathways (discussed below in detail) that regulate proliferation and migration of endothelial cells (Shibuya, Claesson-Welsh 2006). VEGFR3 (Flt-4) is expressed in all endothelia during development, but in the adult it becomes mainly confined to and regulates the lymphatic endothelial cells (Shibuya, Claesson-Welsh 2006, Kaipainen et al. 1995). VEGFR3 is also upregulated in the microvasculature of tumors and wounds (Valtola et al. 1999, Paavonen et al. 2000). Recently it has been shown that VEGFR3 is highly expressed in angiogenic sprouts, and that blocking of VEGFR3 with monoclonal antibodies decreased angiogenesis in a mouse model (Tammela et al. 2008). In the study by Tammela and coworkers, simultaneous use of antibodies against both VEGFR2 and VEGFR3 was shown to result in additive inhibition of angiogenesis and tumor growth, suggesting that targeting VEGFR3 could offer additional efficacy for anti-angiogenic therapies, with the main emphasis on vessels resistant to VEGF or VEGFR2 inhibitors (Tammela et al. 2008).

VEGFs A, C and D are capable of binding to VEGFR2, but VEGFR2 is the main mediator of several physiological and pathological effects of VEGF-A on endothelial cells. These effects include survival via the Akt/PKB –pathway, permeability via nitric oxide production, migration via focal adhesion turnover and actin remodelling, and proliferation through activation of the classical Ras/ERK pathway (Holmes et al. 2007).

There are several autophosphorylation sites on tyrosine residues in VEGFR2, including residues 951, 996, 1054, 1059, 1175 and 1214. Phosphorylation of Tyr951 in the kinase insert domain allows binding and tyrosine phosphorylation of the signalling molecule T-cell specific adapter (TSAd) (Matsumoto et al. 2005). TSAd, containing SH2- and PTB-domains, associates with the cytoplasmic tyrosine kinase Src, leading to regulation of actin stress fiber organization and migratory responses of endothelial cells to VEGF-A. The functional significance of Tyr996 is not clear (Olsson et al. 2006). Tyr1054 and Tyr1059, located in the activation loop of the kinase domain, are critical for the catalytic activity of the receptor (Claesson-Welsh 2003). Phosphorylated Tyr1175 provides a binding site for many signalling proteins, such as phospholipase Cγ (PLCγ), and adaptor proteins Shb and Sck (Holmes et al. 2007). PLCγ in turn stimulates the protein kinase C (PKC) pathway by the generation of inositol trisphosphates and calcium (Shibuya, Claesson-Welsh 2006). Tyr1214 triggers the p38 MAPK cascade via unknown intermediates (Olsson et al. 2006). Thus, VEGFR2 and EGFR signalling have two pathways in common, Akt and ERK.

2.1.4.2. Angiogenesis

Angiogenesis, the formation of new blood vessels from the existing vasculature, is a fundamental process in normal and pathological conditions. It is an intricate chain of events such as activation, chemotactic invasion and migration, morphological change, proliferation and capillary tube formation of ECs from pre-existing blood vessels (Yue et al. 2007). It has been shown that the ECs themselves express all the information necessary to construct a vascular network. The majority of the vasculature is quiescent in normal physiological conditions, with only 0,01 % of the ECs being subject to active cell division (Folkman, Shing 1992, Risau 1997). In adults, angiogenesis occurs during wound healing and menstruation in females. In cancer, angiogenesis is absolutely essential for the growth of solid tumors beyond 1-2 mm in diameter. It has been shown that tumor cells situated within approximately 110 μm of a blood vessel are viable, but beyond this distance from oxygen and nutrient support the cells start to die (Hlatky, Hahnfeldt & Folkman 2002).

VEGF-A is considered to be the most important molecule controlling blood vessel morphogenesis. It has an essential role in the chemotaxis and differentiation of endothelial precursor cells, endothelial cell (EC) proliferation, the assembly of ECs into vascular structures (vasculogenesis) and angiogenic remodelling (Adams, Alitalo 2007). Binding

of VEGF-A to VEGFR2 induces EC differentiation, proliferation and sprouting. Also other factors affect angiogenesis. These include components of the ECM and basement membrane (Cao 2001), such as arresten, canstatin, and endostatin (Nyberg, Xie & Kalluri 2005). Furthermore, physical contact between ECs and pericytes, specialized mesenchymal cells supporting the vessels, is considered to induce a quiescent, non-sprouting phenotype (Bergers, Song 2005).

Sprouting

Angiogenesis is involved in a number of pathological conditions, with VEGFR2 signalling being implicated in tumor angiogenesis and diabetic retinopathy, damage of the retina caused by complications of diabetes mellitus and ultimately leading to blindness (Folkman 2007). The neovascularisation starts when the pro-angiogenic factors outpace the anti-angiogenic factors in a tissue, leading to sprouting of microvascular vessels which eventually organise into new blood vessels (Hanahan, Folkman 1996). Sprouting angiogenesis is an invasive process that requires degradation of the endothelial basement membrane, and removal of matrix proteins from the path of the migrating cells. Also, space must be made in the ECM to allow endothelial cells to form a proper lumen (van Hinsbergh, Koolwijk 2008). For these actions, proteolytic activities by the matrix metalloproteinases (MMPs) and ADAMs (a disintegrin and metalloproteinase domain) are required (van Hinsbergh, Engelse & Quax 2006, Egeblad, Werb 2002). In addition, these proteases also have other roles in angiogenesis, such as activation and modification of growth factors, cytokines and receptors, and the generation of matrix protein fragments that inhibit angiogenesis (van Hinsbergh, Engelse & Quax 2006, Egeblad, Werb 2002, Page-McCaw, Ewald & Werb 2007, Kalluri 2003).

When new vessels are growing, some ECs within the capillary vessel wall are selected for sprouting. These are the tip cells, leading the growing sprout. When the conditions are suitable for angiogenesis, some ECs do sprout, and others fail to respond. Sprouting requires the flipping of apical-basal polarity, the induction of motile and invasive activity, the modulation of cell-cell contacts and local matrix degradation (Adams, Alitalo 2007). VEGF gradients guide the growing EC sprout and pericytes are attracted to the new sprouts by PDGF-BB that is excreted by the tip cells. When the tip cells eventually encounter each other, they fuse. Stalk cell lumen formation involves the fusion of vacuoles formed inside the stalk cells, but there might also be other mechanisms (Adams, Alitalo 2007). Stalk cell proliferation seems to be controlled by VEGF distribution, whereas the tip cell formation following VEGF stimulation is controlled by Dll4/Notch1 signalling (Gerhardt 2008). Delta-like 4 (Dll4) is the ligand of Notch1, type I transmembrane glycoprotein, activatable via direct interaction with transmembrane ligands expressed on the surface of neighboring cells (Bresnick et al. 2000). Thus, the tip and stalk cells have very different functions and it seems that signalling needs to be regulated fundamentally differently between the two cell types.

Anti-angiogenic therapies

As already mentioned, tumors need vasculature to grow beyond the size of approximately 1 mm in diameter. In the search for novel anti-cancer therapies, anti-angiogenic targeting of the neovasculature within tumors is considered a very promising strategy aside the so far used methods of radiotherapy, surgery, and chemotherapy. The angiogenesis inhibitors in clinical use at the moment are bevacizumab (Avastin), a ligand-trapping monoclonal antibody against VEGF-A, and two kinase inhibitors sorafenib (Nexavar) and sunitinib (Sutent), targeting VEGFR tyrosine kinases, mainly VEGFR2 (Bergers, Hanahan 2008). Bevacizumab is used to treat colon cancer, non-small-cell lung cancer and breast cancer, in combination with chemotherapy. Sorafenib and sunitinib are used in treating renal carcinoma, a very angiogenic type of tumor (Bergers, Hanahan 2008).

At present, most anti-angiogenic therapies inhibit VEGF-A and VEGFR2 signalling (Ellis, Hicklin 2008). This has been shown to be successful in some patients, however anti-angiogenic therapy has some challenges that need to be overcome (Jain et al. 2006). First, a considerable portion of cancer patients is resistant to VEGF-based therapies (Burris, Rocha-Lima 2008). Second, even in patients who at first show stabilization of the disease, tumors eventually escape and relapse. As the result, the survival gain of patients treated with anti-VEGF therapies is months rather than years (Bergers, Hanahan 2008, Hurwitz et al. 2004). Third, agents targeting VEGF-A and VEGFR2 cause several side effects impairing quality of life, and can even lead to life-threatening conditions. One reason for this is that in addition to malignancies, VEGF-A is produced in large amounts in healthy conditions to maintain endothelial cell homeostasis (Rudge et al. 2007). For such reasons, individuals with ischaemic heart and brain disease, children and pregnant women must be excluded from this therapy (Verheul, Pinedo 2007). In contrast to VEGFR2, the expression of VEGFR1 and its two ligands VEGF-B and placental growth factor PlGF is increased in various tumors and correlates with progression of the disease and can predict poor prognosis, metastasis and recurring disease in humans. Because of this, a combination therapy targeting PlGF, VEGF-B and VEGFR1 as well as VEGF-A and VEGFR2 could prove useful. Indeed, this combination therapy has now passed phase I clinical trials (Fischer et al. 2008).

One alternative, possibly complementary therapy against cancer is targeting the already established tumor vasculature (Neri, Bicknell 2005). There are differences in the tumor endothelium and stroma surrounding it compared to those of normal tissue. Tumor vasculature is highly disorganized and tortuous, as opposed to the highly organized vessels of normal tissue. In tumor vasculature hierarchy between vessels is low or missing, vessels can be blind-ended, and the intratumor vascular density is heterogenous (Konerding, Fait & Gaumann 2001).

Among other anti-cancer drugs that affect angiogenesis are the microtubule-targeting agents, MTAs (Pasquier, Andre & Braguer 2007). They are divided into microtubule-destabilising agents, including Vinca-alkaloids (e.g. vincristine and vinblastine), and microtubule-stabilising agents, the taxanes (e.g. paclitaxel and docetaxel). The MTAs interfere with tubulin function by binding to the β subunit of α/β tubulin and disrupting microtubule dynamics in tumor cells, ultimately leading to mitotic block and subsequent cell death. They are capable of both inhibiting tumor angiogenesis and directly disrupting the tumor blood vessels: recently the MTAs have raised interest as potential tumor-selective, anti-angiogenic and vascular-disrupting agents (Pasquier, Andre & Braguer 2007). Indeed, several comparative studies have shown that MTAs belong to the most anti-angiogenic chemotherapeutic agents, with human endothelial cells being highly sensitive to them (Hayot et al. 2002, Wang et al. 2003). One of the MTAs, docetaxel, blocked the VEGF-signalling pathways downstream of VEGFR2 at non-cytotoxic concentrations, and not surprisingly inhibited HUVEC migration. Through inhibiting focal adhesion kinase FAK phosphorylation it inhibited both paxillin phosphorylation and αvβ3 integrin activation (Murtagh, Lu & Schwartz 2006).

The intensity of angiogenesis varies between different tumor types. Remarkably, in even the most angiogenic tumors, angiogenesis was 4-20 times weaker when compared with the physiological angiogenesis in the cyclic ovarian corpus luteum, one of the few organ sites in the adult with significant physiological angiogenesis (Eberhard et al. 2000). Also varying levels of pericyte recruitment to the tumor microvasculature were observed in different tumor types, indicating differences in the functional status of the tumor vasculature in different tumors (Eberhard et al. 2000). These results suggest that different tumor types and even tumors within one type, are variably suitable for anti-angiogenic therapies. Tumors in which the levels of angiogenesis are low, are not likely to profit much from therapies depending on endothelial cell proliferation rate. Patient profiling thus plays a significant role in choosing the most suitable therapy for the individual patient: angiogenic status should be determined to identify the patients that will benefit most from anti-angiogenic therapy (Eberhard et al. 2000).

2.1.5. Platelet-derived growth factor receptor β

In mammals there are four different platelet-derived growth factors (PDGFs), namely PDGF-A, -B, C- and –D. The PDGF family of growth factors stimulate cell growth, motility and survival (Heldin, Ostman & Ronnstrand 1998). They belong to the same PDGF/VEGF –family of growth factors as the VEGFs. All PDGFs are dimers of disulfide-linked polypeptide chains, mostly forming homodimers. One heterodimer, PDGF-AB, has been detected in human platelets, but its physiological significance remains unclear (Andrae, Gallini & Betsholtz 2008). After dimerization the PDGFs bind to two RTKs, PDGFRα and PDGFRβ, inducing their dimerization and initiating signalling. Each chain

of the PDGF dimer binds to one receptor subunit. Based on cell culture experiments, multiple possible PDGF-PDGFR interactions exist: PDGF-AA has been shown to form PDGFRαα –dimers, PDGF-AB both PDGFRαα- and PDGFRαβ-dimers, and PDGF-BB to form all three different combinations of PDGFR-dimers (Persson et al. 2004a). The more recently discovered PDGF-dimers CC and DD (Bergsten et al. 2001, LaRochelle et al. 2001, Li et al. 2000) have been demonstrated to signal preferentially via PDGFRαα and PDGFRββ receptor dimers, respectively, but may also activate both types of receptors in cells coexpressing α and β receptors (LaRochelle et al. 2001, Gilbertson et al. 2001). However, according to Andrae and coworkers, in vivo only a few interactions have been shown: PDGF-AA and PDGF-CC acting through PDGFRα and PDGF-BB acting through PDGFRβ (Andrae, Gallini & Betsholtz 2008).

The different PDGFs are synthesized by several different cell types. PDGF-A and –C are expressed in epithelial cells, muscle, and neuronal progenitors. Expression of PDGF-B occurs primarily in vascular endothelial cells, megakaryocytes, and neurons. PDGF-D expression is not very well known, but it has been detected in fibroblasts and smooth muscle cells. Increase in synthesis is often detected in response to external stimuli, such as exposure to low oxygen tension (Kourembanas et al. 1997), thrombin (Daniel et al. 1986, Harlan et al. 1986), or stimulation with different cytokines and growth factors. Of the receptors, both PDGFRα and PDGFRβ are expressed in mesenchymal cells, the latter particularly in vascular smooth muscle cells and pericytes (Andrae, Gallini & Betsholtz 2008). The PDGFRα is involved in gastrulation, and in the development of the cranial and cardiac neural crest, gonads, lung, intestine, skin, central nervous system and skeleton. The PDGFRβ in turn is involved in blood vessel formation and early hematopoiesis (Andrae, Gallini & Betsholtz 2008).

Increased PDGF signalling is linked to a range of diseases. Autocrine activation of PDGF signalling pathways is involved in specific gliomas, sarcomas, and leukemias. Paracrine PDGF signalling occurs commonly in epithelial cancers, triggering stromal recruitment and being involved in epithelial-mesenchymal transition, thereby affecting tumor growth, angiogenesis, invasion, and metastasis. PDGFs promote pathological mesenchymal responses in vascular disorders like atherosclerosis, restenosis, pulmonary hypertension, and retinal diseases, as well as in several fibrotic diseases (Andrae, Gallini & Betsholtz 2008).

Key downstream mediators of the PDGFR signalling include Ras/ERK, PI3K/Akt and PLCγ pathways. The Ras/ERK pathway proceeds as described above for EGFR, through the adaptor proteins Grb2 and Shc, leading to stimulation of cell growth, differentiation, and migration (Andrae, Gallini & Betsholtz 2008). The activation of the PI3K pathway by PDGFRs promotes actin reorganization, directed cell movements, stimulation of cell growth, and inhibition of apoptosis (Hu et al. 1995). The binding of PLCγ to PDGFRs

leads to mobilization of intracellular calcium ions and the activation of PKC (Berridge 1993), resulting in stimulation of cell growth and motility (Kundra et al. 1994).

Various other signalling molecules are engaged by the PDGFRs, including enzymes, adaptors and transcription factors (Andrae, Gallini & Betsholtz 2008). For instance, activation of tyrosine kinase Src leads to Myc transcription and mitogenic responses, adaptor proteins Nck and Crk bind to PDGFRs via their SH2-domain and are involved in activation of Jun amino-terminal kinase (JNK) (Nishimura et al. 1993, Su et al. 1997). Adaptor protein Grb7 has a SH2 domain and binds to Tyr775 of PDGFRβ but its role in PDGFRβ signalling is unclear (Yokote et al. 1996, Ronnstrand, Heldin 2001). Also STAT transcription factors may bind to PDGFRs, phosphorylating and activating them (Darnell 1997).

Integrin αvβ3 interacts with the PDGFRβ as well, enhancing cell proliferation, migration, and survival (Frisch, Ruoslahti 1997). This interaction aids in localizing PDGFRs and interacting molecules at focal adhesions, sites where integrins cluster together and where several signalling pathways initiate and cross-talk (Clark, Brugge 1995).

2.1.6. Met / Hepatocyte growth factor receptor

Met is the ubiquitously expressed RTK for hepatocyte growth factor (HGF) / scatter factor (SF). The Met heterodimer consists of a single-pass transmembrane β chain (145 kDa) and a fully extracellular α chain (50 kDa). It has a crucial role in the control of so called invasive growth, a complex process involving cell-cell dissociation, anchorage-independent growth and branching morphogenesis (Giordano et al. 2002). Met controls the normal function and homeostasis of several organs by regulating cell proliferation, differentiation and migration both during embryogenesis and organ regeneration in the adult (Bladt et al. 1995, Borowiak et al. 2004, Huh et al. 2004). Genetic analysis of HGF and Met has proven that this ligand-receptor pair is essential during development to be able to control processes that are specific to vertebrate, or even mammalian, embryogenesis (Birchmeier et al. 2003). Met belongs to the same RTK family as highly homologous Ron (Ronsin et al. 1993). Also the ligand for Ron, macrophage-stimulating protein, shows high homology to HGF as well as plasminogen. Together the two growth factors constitute a family with related biological activities, called plasminogen-related growth factors (Peschard, Park 2007).

The activation of Met proceeds as described for the RTKs in general (see above). In addition to the Ras/ERK and Akt/PKB –pathways in common with the abovementioned EGFR, VEGFR2 and PDGFRs, Met signalling controls Pak- and Rap1-pathways that have roles in cell polarity, motility, migration and invasion (Birchmeier et al. 2003). Tyrosine phosphorylation sites on Met include Tyr1349 and Tyr1356 in the carboxy-terminal docking site, Tyr1234 and Tyr1235 in the kinase domain and Tyr1003 located in the juxtamembrane

domain (Birchmeier et al. 2003). The twin tyrosines 1349 and 1356 form a unique, bidentate multisubstrate binding site conserved in Met family members. When active, this site binds substrates such as Gab1, Grb2, and PI3K (Ponzetto et al. 1994, Weidner et al. 1996). Only one substrate can bind at a time to a Met monomer due to steric hindrance. Grb2 and PI3K attach to phosphotyrosines on Met via their SH2-domains, while Gab1 uses its Met-binding site, a sequence of 13 amino acids, to bind Met. This characteristic is unique to Gab1 and results in direct and robust interaction with Met, and a prolonged Gab1 phosphorylation in response to HGF. Phosphorylated Gab1 subsequently binds to SHP2, PI3K, PLC, and Crk via their SH2-domains, leading to activation of ERK/MAPK, Akt/PKB and Rap1 –pathways and thus to effects in cell proliferation, cell-cycle progression, migration, invasion and survival (Birchmeier et al. 2003).

In cancers, Met receptor is deregulated resulting in progression of the disease, invasive behaviour and metastasis formation (Kermorgant, Parker 2005). Interestingly, the Met receptor is predominantly expressed in epithelial cells, while its ligand HGF is expressed in mesenchymal cells (Peschard, Park 2007). Ligand (HGF) stimulation, receptor amplification, or constitutive activation of the receptor induce cancer cells to leave the primary tumor, invade through the basement membrane, move towards target organs and there give rise to metastasis (Giordano et al. 2002, Trusolino, Bertotti & Comoglio 2001).

Not surprisingly, several therapeutic studies have been targeted at blocking HGF and Met function in cancer (Ma et al. 2003). Encouraging evidence obtained during the few most recent years allows for proposing Met as an optimal target in anti-cancer therapy. First, Met has been shown to have a central role in cancer formation and metastasis. Second, somatic mutations in Met coding sequence or increase in copy number has been observed in certain human cancers (Benvenuti, Comoglio 2007). Since invasion and metastasis are the main causes of death for cancer patients, therapeutic approaches targeted at impairing metastasis are needed. Hence Met and its ligand HGF represent valid targets. Several strategies are used to neutralize the Met receptor in vitro and in vivo: ligand antagonists such as anti-HGF monoclonal antibodies, uncleavable HGF and NK4 (a competitive antagonist of HGF-Met association), kinase inhibitors, and receptor competitors (e.g. anti-Met monoclonal antibodies), competitor peptides, and shRNA (Benvenuti, Comoglio 2007, Heideman et al. 2004, Matsumoto, Nakamura 2003). With the present knowledge, most likely anti-Met therapeutics will be in clinical use for treating cancer in the future.

2.1.7. Insulin receptor

The insulin receptor (IR) is a transmembrane receptor like the other RTKs and is activated by insulin. In contrast to the rest of the RTKs, IR is a dimer in its inactive form and complexes into a tetramer in response to ligand binding. It consists of two α and two β subunits, the former of which are mainly extracellular, and the latter mainly intracellular.

The β subunits are linked by disulfide bonds and also contain the tyrosine kinase domain (Pollak 2008). Insulin receptors are widely expressed in normal tissues.

Insulin is a key regulator of energy metabolism and growth, being the predominant carbohydrate metabolism regulator. It differs from many other cancer-relevant regulatory peptides such as EGF and PDGF in that it regulates physiology at both the level of the whole organism and at the cellular level (Pollak 2008). Insulin is produced by the pancreatic β-cells in response to increasing glucose concentrations in the blood, and it reaches its target tissues through circulation. Similar to RTKs in general, insulin binds to the extracellular α-subunits, triggering a conformational change that activates the intrinsic tyrosine kinase activity of the intracellular β-subunit via transphosphorylation of specific tyrosine residues in the activation loop. IR-substrates (IRS) bind to the phosphotyrosines and in turn become phosphorylated by IR (Asante-Appiah, Kennedy 2003). Functioning as adaptor proteins, the IRSs subsequently activate PI3K, the SH2 / SH3 adaptor proteins Grb2 and Nck, and SHP2, leading to proceeding of Akt-, Ras/ ERK- and mTOR –signalling (Asante-Appiah, Kennedy 2003, Goldstein et al. 1998b).

Impaired insulin action causes diabetes, which is caused by diminished or lack of production of insulin in the pancreas (type I diabetes) or by lowered ability to use the produced insulin (type II diabetes). Type II diabetes affected 6 % of the world's adult population in 2006 and the incidence is predicted to rise to 7 % by the year 2020 (Muoio, Newgard 2008). Insulin and the insulin receptor are not only involved in normal tissue metabolism. Recent studies suggest that the IR is commonly expressed in human tumors (Cox et al. 2009, Law et al. 2008, Frasca et al. 2008), where it functions as an important activator of the Akt and ERK/MAPK –signalling networks. Increased insulin production seems to be associated with worse cancer prognosis (Pollak 2008). Regulation of IR and understanding its functions are thus important also in terms of drug development.

2.1.8. Colony stimulating factor 1 –receptor

Colony-stimulating factor-1 (CSF-1, also called macrophage-CSF) is the main regulator of the survival, proliferation, differentiation and function of mononuclear phagocytes (Chitu, Stanley 2006) and also an attractive therapeutic target for malignant diseases and chronic inflammation (Irvine et al. 2006). In early myeloid differentiation, CSF-1 synergizes with other growth factors to produce mononuclear phagocyte progenitor cells (Pixley, Stanley 2004). Later, CSF-1 can itself regulate the differentiation and proliferation of mononuclear phagocyte progenitor cells to monocytes and macrophages (Stanley, Chen & Lin 1978, Cecchini et al. 1994). Not surprisingly, mice lacking either CSF-1 or its receptor have severely reduced amounts of macrophages in vivo (Simoncic et al. 2006).

CSF-1 transmits its effects by binding to the CSF-1-receptor, thereby stabilizing the dimerization of the receptor. The cytokine binds to the extracellular region composed

of five immunoglobulin-like domains (Douglass et al. 2008). The subsequent autophosphorylation after CSF-1 binding initiates tyrosine phosphorylation cascades in the cell, leading to rapid stimulation of cytoskeletal remodelling, gene transcription and protein translation (Yeung, Stanley 2003). There are at least eight phosphotyrosines in cytoplasmic portion of CSF-1R, namely Tyr546, Tyr561, Tyr699, Tyr708, Tyr723, Tyr809, Tyr923, and Tyr969. Following dimerization of two CSF-1R monomers, autophosphorylation of the tyrosines occurs within minutes by the internal kinase domain of the receptor (Li, Stanley 1991). The phosphorylated tyrosines attract several linker proteins such as PI3K, Grb2, Src, Cbl and monocyte adaptor Mona, leading to effects in cell proliferation, survival, differentiation, cell adhesion, spreading, polarization, motility and phagocytosis (Pixley, Stanley 2004).

Like in the cases of the five abovementioned RTKs, the signalling downstream of the CSF-1R proceeds along the Akt/PKB and Ras/ERK –pathways. The latter represents the dominant mitogenic pathway in most types of non-hematopoietic cells in the human body. Not surprisingly therefore, it is frequently involved in human cancers in deregulated form. Together the two pathways promote cell proliferation, survival, migration and invasion (Weinberg 2007).

2.2. Protein tyrosine phosphatases

2.2.1. Definition and general characteristics

Whereas the PTKs originate from a common ancestor, the protein phosphatases have evolved in separate families having structurally and mechanistically distinct features (Tonks 2006). The serine/threonine phosphatases (S/TPs), another group belonging to the protein phosphatases in addition to the PTPs, exist in vivo as a variety of holoenzyme complexes, consisting of multiple combinations of catalytic and regulatory subunits that control a broad spectrum of signalling pathways (Cohen 2004). The S/TPs function as catalysators in the direct hydrolysis of a phospho-substrate, with two metal ions in the active site of the enzyme facilitating the reaction (Tonks 2006). Another example of the structural and mechanistic variability among the protein phosphatase families is the group of haloacid dehalogenases, which have essential aspartic acid residues in the active site instead of the tyrosine or serine/threonine. This group includes phosphatases such as Eyes absent (Eya) that also has a role as a transcription factor (Epstein, Neel 2003) and chronophin which affects the dynamics of the actin cytoskeleton (Wiggan, Bernstein & Bamburg 2005). There are also phosphatases that control signal cascades through effects on non-proteinous substrates. One of these is PTEN, phosphatase and tensin homolog deleted on chromosome 10, which dephosphorylates phosphoinositide phosphates (PIP). PTEN activity is lost in 30-40 % of all human cancers, such as glioblastoma, endometrial, prostate, breast and thyroid carcinomas. PTEN is the second most frequently mutated

gene in human cancer after p53 (Yin, Shen 2008). Its absence leads to accumulating levels of phosphorylated PIP3 and consequently to increased Akt signalling, which in turn downregulates e.g. Death Receptor and tumor suppressor p53 function, the latter via MDM2, the predominant negative regulator of p53 (Volk et al. 2009). Belonging to the dual-specificity phosphatases, PTEN is also able to dephosphorylate phosphotyrosine (Yamada, Araki 2001). Other examples of phosphatases controlling signalling cascades via affecting non-proteinous substrates include Src homology-2 (SH2) domain containing inositide phosphatases (SHIPs) and synaptojanins, which catalyse the dephosphorylation of inositol phospholipids (Hughes, Cooke & Parker 2000).

The first PTPs, PTP1A and B, were purified in 1987 (Tonks, Diltz & Fischer 1988b, Tonks, Diltz & Fischer 1988a), years after the first PTK, non-receptor tyrosine kinase Src (Czernilofsky et al. 1980). At the time it had recently been discovered that the transforming proteins of certain acutely oncogenic retroviruses and receptors for several hormones and growth factors have intrinsic tyrosine kinase activity (Sasaki et al. 1985, Hunter, Cooper 1985). This suggested an important role for the PTKs in the regulation of cell proliferation, but the structure and regulation of the counter-enzymes, the PTPs, was very unclear (Tonks, Diltz & Fischer 1988b). The first PTPs were identified in human placenta, using a novel artificial substrate, namely reduced, carboxamidomethylated and maleylated lysozyme. Both ~35 kDa enzymes contained PTP catalytic subunits and were specific for phosphotyrosyl residues, leaving phosphoseryl and –threonyl residues intact (Tonks, Diltz & Fischer 1988b, Tonks, Diltz & Fischer 1988a). The first crystal structure of a PTP, namely PTP1B, was solved before the first PTK structure, only 6 years after reporting the identification of the first PTP (Barford, Flint & Tonks 1994).

The unifying feature among all PTP family members is the active site sequence motif HCX_5R, in which the cysteine residue functions as a nucleophile and is essential for catalysis (Tonks 2006, Guan, Dixon 1991). The motif is often referred to as the PTP signature motif or the PTP loop, and it resides in the conserved catalytic domain of approximately 280 residues. The conserved structure of the PTP loop allows for the catalytic Cys and the arginine, involved in phosphate binding and in stabilisation of the reaction intermediate, to stay in close proximity, thus forming a nest to hold the phosphate group of the substrate in place for nucleophilic attack (Tabernero et al. 2008, Zhang, Wang & Dixon 1994). Due to the microenvironment of the PTP loop, the cysteine residue has a low pKa, and this makes it vulnerable to oxidation (Zhang, Dixon 1993, Peters, Frimurer & Olsen 1998). Residues of this motif form the phosphate-binding loop, located at the base of the active site cleft. The depth of the cleft is likely to strongly contribute to the specificity for phosphotyrosine-containing peptides, since the smaller phosphoserine and –threonine side chains are not long enough to reach to the phosphate binding site (Barford, Flint & Tonks 1994). In addition, a secondary substrate-binding pocket has been observed in PTPs. Initially, it was found in PTP1B (Barford, Flint &

Tonks 1994), but a recent study by Barr and coworkers revealing the crystal structures of 22 human PTPs showed that this structure is present in most PTPs (Barr et al. 2009). A small gateway region separates the secondary substrate-binding site from the primary, catalytic pocket. This gateway region consists of a direct groove between the catalytic pocket and the secondary substrate binding site that is flanked by two bulky residues. The gateway region and the secondary substrate binding site were analysed in different PTPs by Barr and others and as a result most PTPs were divided into five groups, based on whether the two elements were "open" or "closed" (Barr et al. 2009, Tremblay 2009). Both the gateway region and the secondary substrate binding site can be expected to be crucial for substrate specificity, and thus also important in development of possible PTP-inhibitors (Barr et al. 2009, Tremblay 2009). Importantly, such PTP1B inhibitors already exist that simultaneously block both the catalytic pocket and the secondary substrate-binding pocket (Zhang, Zhang 2007). The PTPs are also characterised by N- or C-terminal noncatalytic segments that frequently serve regulatory functions such as directing the protein to a specific subcellular location.

Protein tyrosine kinases are encoded by 90 genes, whereas protein tyrosine phosphatases are encoded by 107 genes in the human genome (Alonso et al. 2004, Barr et al. 2009). Of these 107, 11 are catalytically inactive, 2 dephosphorylate mRNA, and 13 dephoshorylate inositol phosholipids. As a result, 81 PTPs are active, with the capability of dephosphorylating phosphotyrosine. Of the 90 PTKs, 85 are considered to be catalytically active. Thus, there are approximately as many PTPs and PTKs, and therefore also their substrate specifities could be similar (Alonso et al. 2004). This suggests similar levels of complexity between the two families. However, further diversity is provided by the use of alternative promoters, alternative mRNA splicing and post-translational modifications (Tonks 2006). Redundancy between the different PTPs is likely at least in some cases, since the consequences of PTP loss range from early embryonic death to mild or no effects (Hendriks et al. 2008).

PTPs display exquisite substrate, and functional, specificity in vivo. This is reflected by the fact that despite the largely conserved fold, the surface properties of the PTPs are strikingly diverse (Barr et al. 2009). The PTPs control various functions such as focal adhesion dynamics, cell-cell adhesion and signalling of various PTKs. On the other hand, PTPs are regulated by phosphorylation, dimerization, and reversible oxidation (Stoker 2005). As oncogenic activation of several PTKs plays a significant role in cancer, PTPs as PTK counter-actors have emerged as highly interesting targets for drug development. At least 30 PTPs have been shown to play a role in cancer, in addition to metabolic, muscle, neurological, and autoimmune diseases (Alonso et al. 2004, Andersen et al. 2004).

As always when trying to elucidate the functions of a protein, one should investigate the actions of the particular protein in conditions that are as close to physiological as

possible. Comparing animal model data with in vitro data brings valuable information about the differences: the data emphasizes the importance of investigating PTP action under close-to-physiological conditions (Hendriks et al. 2008). This might in part be due to the fact that PTPs (via active site cysteine) are differentially oxidized and inactivated in vitro and in living cells (den Hertog, Groen & van der Wijk 2005).

2.2.2. PTP classification

The PTPs are divided into classical, phosphotyrosine-specific phosphatases and the dual specificity phosphatases (DSPs) (Tonks 2006). All PTPs have at least one catalytic domain, containing the signature motif and within it a conserved cysteine residue that is necessary for phosphatase activity (Andersen et al. 2001). The classical PTPs, comprising 38 members, can be further divided into receptor-PTPs (rPTP) with 21 members, and non-receptor-PTPs (nrPTP) with 17 members (Bauler, Hendriks & King 2008). The rPTPs reside in the plasma membrane, while the nrPTPs are localised to various intracellular compartments, like the cytosol, endoplasmic reticulum, and the proximity of plasma membrane (Stoker 2005). The DSPs comprise the most heterogenous group among the PTPs in terms of substrate specificity, with the ability to dephosphorylate phosphotyrosines, -serines, and –threonines. This review concentrates on the classical PTPs (Figure 4), and in more detail on the nrPTP T-cell protein tyrosine phosphatase, TCPTP.

2.2.2.1. Domain structure of rPTPs

The rPTPs have a general structure of a membrane receptor, with an extracellular domain, a single transmembrane segment and one or two conserved PTPase catalytic domains (D1 and D2) in tandem. All the activity resides in the membrane-proximal PTP domain (D1), and with the exception of rPTPα, the membrane-distal PTPase catalytic domain (D2) is indeed inactive ("PTP pseudo domain" in Fig. 4) (Tonks 2006, Buist et al. 1999). However, the D2 has an important role in the activity, specificity and stability of the rPTP (Felberg, Johnson 1998, Streuli et al. 1990), and in controlling protein-protein interactions regulating rPTP dimerization (Blanchetot et al. 2002, Jiang, den Hertog & Hunter 2000a). Often the inactive D2 domain partners with the active PTP-domain. For instance, CD45 needs its inactive D2 domain for dephosphorylation of its physiological substrates by the membrane-proximal, active D1 domain (Kashio et al. 1998). Also, D2 is strongly involved with D1 in the head-to-toe –model of rPTP inactivation introduced by Barr and coworkers (Barr et al. 2009), discussed more later. Most rPTPs have extracellular domains such as immunoglobulin-like domains (IG) and fibronectin type III domains (FN), like the extracellular domains of cellular adhesion molecules. They assist in binding of the cell to other cells and to the ECM.
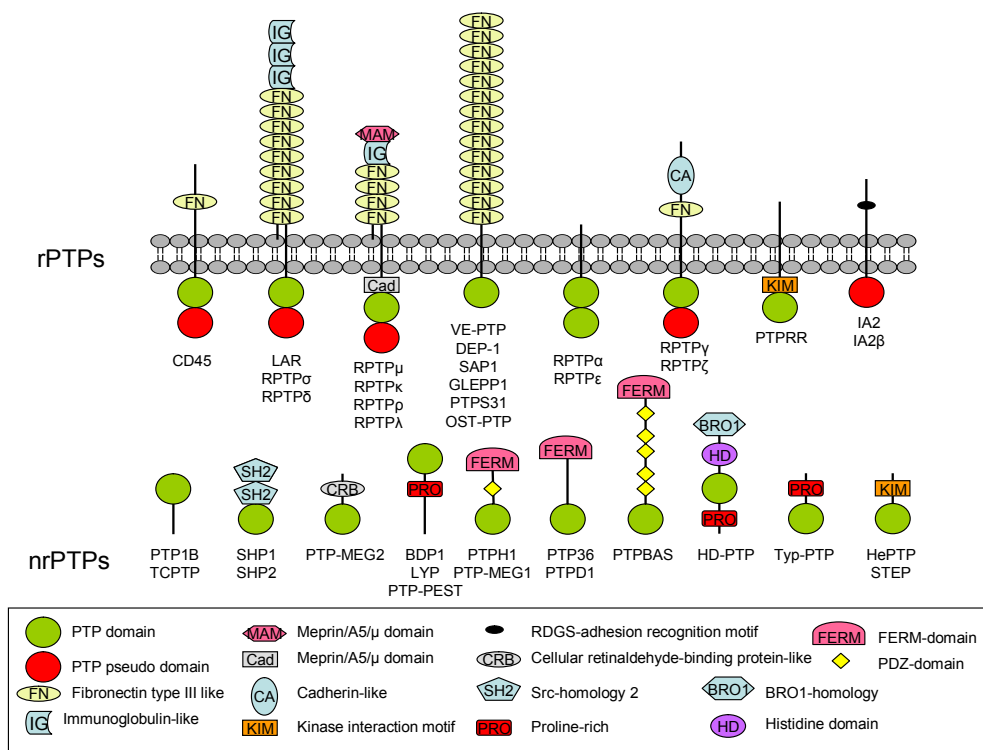
Figure 4. Schematic view of the domain composition of the classical PTPs. Adapted from (Hendriks et al. 2008, Tonks 2006).

2.2.2.2. Domain structure of nrPTPs

The nrPTPs have a single catalytic PTPase domain and additional non-catalytic regions or domains that have roles in regulation of the enzymatic activity, recruiting specific ligands, binding to specific proteins, or targeting them to specific subcellular locations (Alonso et al. 2004, Mauro, Dixon 1994). Each domain is capable of recognizing and binding a specific sequence or structure present in the partner molecule. These domains include membrane-association domains (as in PTP1B), nuclear-localisation domains (as in TCPTP), Src homology 2 (SH2) domains (as in SHP1 and -2) and cytoskeletal-association domains (as in PTPH1) (Mauro, Dixon 1994).

The SH2 domain is a 100 amino acids long, conserved sequence motif that regulates cross-talk between cytoplasmic signalling molecules and RTKs. It binds to phosphotyrosine residues on autophosphorylated RTKs, and brings together multiple components of the appropriate signalling pathway. The SH2 domain was originally found in v-Src family of non-receptor tyrosine kinases but has since been found in a broad variety of proteins. Proline-rich sequences, found in five different nrPTPs (Fig. 4), are expressed widely from prokaryotes to eukaryotes (Li 2005). They are usually C-terminal (canonical) (Sanda et

al. 2008), but can also be internal (non-canonical). The sequences are recognised by a broad array of proline-recognition domains (PRD), which include e.g. the Src homology 3 (SH3) -domain and WW domain (named after two highly conserved tryptophan residues). Like SH2 domains, SH3 domains mediate protein-protein interactions between different signalling components downstream of membrane-bound receptors (Sanda et al. 2008). SH3 domains can be found e.g. in eukaryotic signal transduction and cytoskeletal proteins such as Grb-2 and p130Cas, to which PTP1B binds via its proline-rich sequence (Liu, Hill & Chernoff 1996). In addition to nrPTPs, proline-rich sequences are also found in two members of the dual-specificity phosphatases (Alonso et al. 2004).

FERM-domains (Band 4.1, Ezrin, Radixin, Moesin) are involved in protein – protein and protein – lipid –interactions, often mediating binding of intracellular proteins to cellular membranes via cytoplasmic tails of transmembrane proteins (Chishti et al. 1998, Lim et al. 2008). They are usually located in the N-terminus of the protein. FERM-domains have been identified in several nrPTPs (Fig. 4) (Gu et al. 1991, Hendriks et al. 1995, Moller et al. 1994), the cell-cell contact protein talin (Rees et al. 1990) and focal achesion kinase FAK (Lim et al. 2008). FERM mediates nuclear localization of FAK and promotes cell survival through the inhibition of tumor suppressor p53 activation in development and cellular stress (Lim et al. 2008). The FERM in talin contains several binding sites for β integrins, the cytoplasmic tail of which binds to FAK via talin or paxillin (Ziegler et al. 2008). Also Myosin-X binding to integrins is mediated by FERM-domain (Bohil, Robertson & Cheney 2006).

The PDZ-domain (postsynaptic density-95 – discs large – zonula occludens 1 homology) is found in all species and it is one of the most common protein-protein interaction domains in humans (Jemth, Gianni 2007). PDZ-domains are expressed in various proteins in humans, alone or in arrays. They can be grouped according to their ligand specificity, however displaying overlapping specificity and promiscuity toward their different target ligands (Jemth, Gianni 2007). The PDZ domains help to anchor transmembrane proteins to the cytoskeleton and hold together signalling complexes (Bauler, Hendriks & King 2008, Doyle et al. 1996). The counter proteins (receptors or signalling proteins) of the PDZ domains have specific C-terminal PDZ-binding motifs. Three classical PTPs contain PDZ-domains: PTPH1, PTP-MEG1, and PTPBAS, all belonging to the nrPTPs (Bauler, Hendriks & King 2008).

The cellular retinaldehyde-binding protein like domain, CRB (also called Sec14-homology domain) is present only in PTP-MEG2 and mediates functional association with secretory vesicles (den Hertog, Ostman & Bohmer 2008). The kinase-interacting motif KIM in turn mediates binding to MAPK (den Hertog, Ostman & Bohmer 2008) and among the classical PTPs it is found only in rPTP PTPRR and nrPTPs HePTP and STEP (Fig. 4).

Both the catalytic domain and noncatalytic segments contribute to the substrate specificity of the PTPs. Basically the domain structure determines a subgroup of partners with which

the protein is able to communicate, thus ensuring that signals are passed only to intended targets. The noncatalytic segments target the enzymes to specific subcellular locations (Mauro, Dixon 1994) and affect substrate specificity (Andersen et al. 2001), whereas the catalytic domains have structural features that have effects on the latter (Tonks, Neel 2001). PTP engagement in different signalling networks should not be predicted based on sequence information, since it is still not clear which residues of the catalytic PTP domain contribute to substrate-specificity profiles (Tiganis, Bennett 2007). Andersen and coworkers pondered that no single residue in the nonconserved areas would be unique to any particular PTP, but instead the combination of residues (Andersen et al. 2001).

In addition to substrate specificity and correct subcellular locations, alternative splicing, post-translational modifications, differential expression and correct tissue distribution of PTPs also play crucial roles in enabling proper enzyme function (den Hertog, Ostman & Bohmer 2008). These issues are discussed next.

2.2.3. Regulation of classical PTP action

PTP activity is regulated at different levels, ranging from organ level to the cellular level (Figure 5). Temporal control of PTP activity is present at all levels, as the proteins need to be not only at certain place but also at certain time to be able to perform their actions. Below the different levels are discussed, with emphasis on the molecular level.

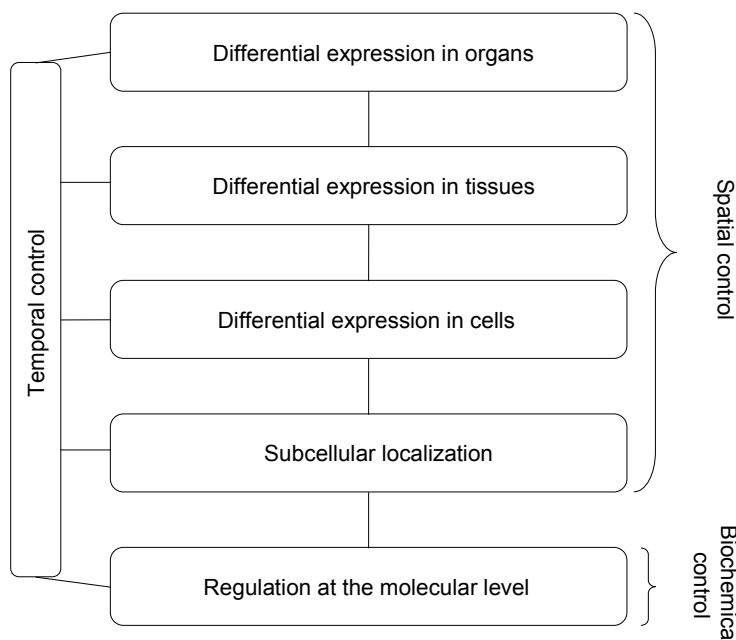**Figure 5. Different levels of PTP regulation.** PTPs are expressed differentially in specific organs, tissues and cells. At the cellular level, PTPs are regulated by directing them to different subcellular locations. Regulation at the molecular level consists of post-translational modifications. Temporal control exists at all the different levels. Modified from (den Hertog, Ostman & Bohmer 2008).

2.2.3.1. Expression

Levels of expression in specific organs differ among the PTPs. Some PTPs are ubiquitously expressed like PTP1B and TCPTP, and others are expressed more selectively, as nrPTP LYP in the hematopoietic compartment (Pao et al. 2007). To enlighten the importance of tissue-specific expression, it is essential that e.g. PTPs involved in insulin receptor (IR) signalling are expressed in insulin-sensitive tissues (Goldstein et al. 1998a). It has been estimated that individual cells express 30-60 % of the entire selection of PTPs and PTKs (Alonso et al. 2004). Tissue-specific regulation of PTP expression is regulated by e.g. alternating use of promoters or exon skipping within the PTP genes, with SHP1 as an example (Banville, Stocco & Shen 1995). Also mRNA stability can be regulated to control PTP expression. For instance, TCPTP but not PTP1B mRNA stability has been observed to have increased in mitogen-stimulated T-lymphocytes (Rajendrakumar, Radha & Swarup 1993). According to den Hertog and coworkers (den Hertog, Ostman & Bohmer 2008), PTP levels are probably also controlled at levels of translation and protein stability. For example, PTPs like SHP2 have a reasonably long half-life, while others like PTPRR have a short half-life (Dilaver et al. 2007, Siewert et al. 1999).

2.2.3.2. Subcellular localization

The various different protein-protein interaction domains and targeting domains in PTPs (see 2.2.2.2. Domain structure of nrPTPs) direct the enzymes to correct locations in the cell. SH2, FERM, and PDZ-domains direct nrPTPs and rPTPs to activated cell-surface receptors such as the RTKs (den Hertog, Ostman & Bohmer 2008). Nuclear localisation signal of e.g. TCPTP 45 kDa form directs it to the nucleus, and the ER targeting domain of the 48 kDa form of TCPTP leads it to the ER. CRB-domain is mediating the functional association with secretory vesicles, kinase interacting motif (KIM) mediates binding to MAPK. The catalytic domain of rPTPs is released into the cytoplasm and maybe also to the nucleus by proteolysis, and specific domains direct PTPs to lipid rafts (den Hertog, Ostman & Bohmer 2008).

2.2.3.3. Regulation at the molecular level

Phosphorylation

Phosphorylation affects catalytic activity of the enzymes either directly by allosteric mechanisms or by arranging binding sites for other proteins. Both rPTPs and nrPTPs are regulated by phosphorylation of their Ser or Thr residues (den Hertog, Ostman & Bohmer 2008). For instance, phosphorylation at C-terminal seryl residues has been observed to be involved in the transition from G2 to M phase of the cell cycle in PTP1B (Flint et al. 1993). Also, it has been shown that Ser304 of TCPTP is phosphorylated in a cell-cycle-dependent manner (Bukczynska et al. 2004). However, this phosphorylation did not lead to changes in activity, protein stability or the location of TCPTP. Bourdeau

and coworkers speculated that the serine-phosphorylation could be a means to regulate TCPTP activity temporally, depending on the stimulus and/or growth factor involved (Bourdeau, Dube & Tremblay 2005).

The PTPs can also be phosphorylated on tyrosines, creating binding sites for proteins containing SH2 –domains that have a specific affinity for phosphorylated tyrosines as explained above in "Domain structure of nrPTPs". For example, the PTK Csk has been shown to associate with PTP BDP1, using its SH2-domain to bind a pTyr on the BDP1 (Wang et al. 2001).

Also SHP2 activation is regulated by phosphorylation. When inactive, the protein consisting of two SH2-domains in the N-terminus and a PTP domain in the C-terminus (Figure 4), is in a closed conformation, the N-terminal SH2-domain resting aside the C-terminus. Addition of a pTyr peptide that can bind the N-SH2 domain "opens" the enzyme and allows access to the active site. Many of the oncogenic mutations of SHP2 are located in the interface between the N-SH2 and the PTP-domain, disrupting this autoinhibitory mechanism. This leads to a lowered threshold in SHP2 activation and consequently to increased and continuous activation of signalling pathways such as Ras/ ERK and Akt (Ostman, Hellberg & Bohmer 2006, Mohi, Neel 2007).

Cleavage

PTPs, like many other proteins, are targets of post-translational proteolytic cleavage. Several rPTPs such as LAR, rPTPμ and rPTPσ are cleaved by subtilisin-like proteases (Campan et al. 1996, Aicher et al. 1997, Streuli et al. 1992). This can lead to ectodomain shedding, at least in cell culture. Occurrence of shedding in vivo is hypothetical, but it could provide a mechanism to terminate rPTP signalling, facilitate internalisation of catalytic domains, or to release ectodomains to compete for ligands (Stoker 2005). Cleavage occurs also inside the cell. For instance, rPTPα, rPTPε and PTP1B can be cleaved in a calpain-dependent manner, releasing their catalytic domains into the cytoplasm (Frangioni et al. 1993, Gil-Henn, Volohonsky & Elson 2001). This can lead into two things: it can block the access of the PTPs to membrane-associated substrates, or clear the way to novel substrates (Stoker 2005).

Oxidation

Reversible oxidation of the catalytic domain invariant cysteine is a means to negatively regulate PTPs. Reactive oxygen species (ROS) are produced as a result of activation of various classes of cell-surface receptors, such as RTKs, integrins, and G-protein-coupled receptors, and are now regarded as important regulators of PTP signalling (Fig. 6) (Salmeen, Barford 2005, Finkel 2003, Bokoch, Knaus 2003, Lambeth 2004). Because of the low pKa of the active site Cys residue, PTPs are very sensitive to inhibition by ROS. On the other hand the low pKa promotes the function of the Cys residue as a

nucleophile in catalysis but then makes it vulnerable to oxidation resulting in transient inhibition of PTP activity (Barrett et al. 1999, Denu, Tanner 1998, Lee et al. 1998). Depending on the extent of oxidation, the Cys in PTP active site can be converted to either sulphenic (SOH), sulphinic (SO$_2$H) or sulphonic (SO$_3$H) acid. For the oxidation to be reversible, the active site Cys must not be oxidized further than sulphenic acid, since higher oxidation is usually irreversible (Fig. 6) (Tonks 2006).
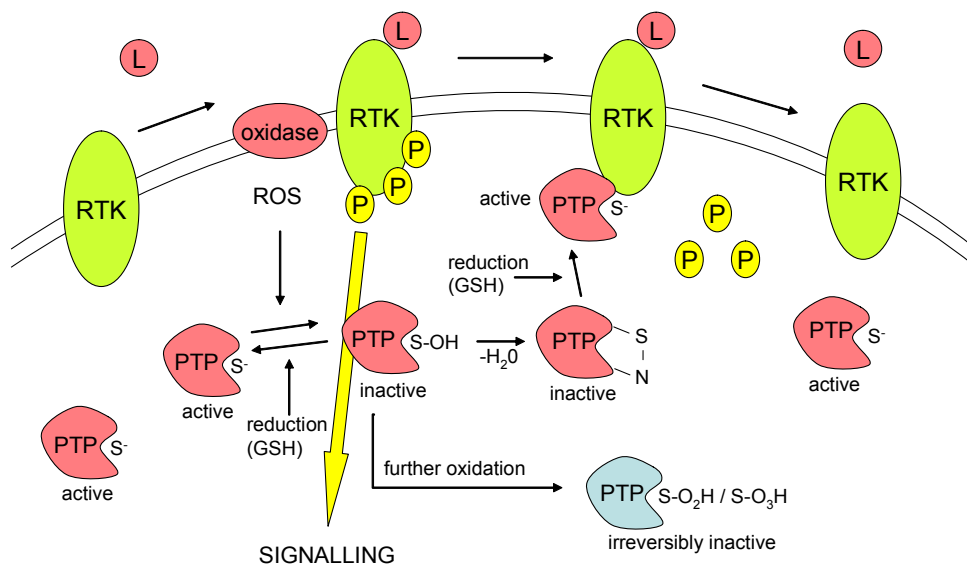
Figure 6. Regulation of PTP activity by oxidation. Following stimulation of an RTK by a ligand, a Rac-dependent multiprotein NADPH oxidase complex ("oxidase") is assembled and activated, leading to production of ROS. The sulfur atom at the PTP active site Cys is usually present as a thiolate ion, promoting its function as a nucleophile, rendering it very sensitive to oxidation. In contact with ROS, Cys is oxidized to sulfenic acid (S-OH), and then rapidly converted to cyclic sulfenamide (-S-N-), resulting in inactive PTP and proceeding of RTK signalling. PTP activity is restored after reducing the active site Cys back to the thiolate form using e.g. reduced glutathione (GSH). The mechanism presented here is used to transiently inactivate PTP during RTK activation. Further oxidation leads to irreversible forms of sulphinic (S-O$_2$H) and sulphonic (S-O$_3$H) acid. Modified from (Ostman, Hellberg & Bohmer 2006, Meng et al. 2004).

Insulin stimulation, creating ROS, has been shown to cause rapid and transient oxidation and inhibition of TCPTP and PTP1B in an in-gel phosphatase assay (Meng et al. 2004). More recent results in pancreatic cancer cells show inhibitory effects of ROS production on a PTP, namely LMW-PTP (Lee et al. 2007). Inhibition of LMW-PTP was shown to lead to enhanced and sustained phosphorylation of kinases, such as PTK Janus kinase

2 (JAK2), and suppression of apoptosis. This pathway mediates the survival-promoting effect of ROSs and suggests new targets for pancreatic cancer treatment (Lee et al. 2007).

Also RPTPs are controlled by oxidation. In RPTPα, the Cys of the signature motif from the second, membrane-distal PTP domain (RPTPα-D2) is more vulnerable to oxidation than the membrane-proximal catalytic domain (Persson et al. 2004b). After oxidation of RPTPα-D2 inside the cell, a conformational change occurs in the extracellular part of RPTPα (van der Wijk et al. 2003). Thus, the membrane-distal PTP domain may function as an oxygen sensor as well as transmit inside-out signalling through RPTPα.

Meng and co-workers speculate that ligand-induced oxidation, required for optimal tyrosine phosphorylation, can be used to "tag" the PTPs which are critical regulators of the signalling response to that particular ligand. In practice the agonist can boost tyrosine phosphorylation directly or indirectly: by activating a RTK, or by inactivating a PTP, respectively. Thereby, ROS produced as a result of agonist (e.g. insulin) stimulation facilitate the initiation of the signalling response by inhibiting the PTP that would otherwise downregulate the RTK in question. This is the case with insulin signalling and TCPTP. It also seems that this regulation mechanism may apply to many PTPs since various ligands have been demonstrated to trigger both tyrosine phosphorylation and ROS production (Meng et al. 2004, Meng, Fukada & Tonks 2002). However, this is not always the case, as PDGF stimulation does not lead to oxidation of TCPTP (Meng et al. 2004) although PDGFRβ has been shown to be a TCPTP substrate (Persson et al. 2004a). In contrast, PDGF induced the transient oxidation and inactivation of a distinct PTP, SHP2 (Meng, Fukada & Tonks 2002), the substrate of which PDGFRβ has been demonstrated to be (Lechleider et al. 1993).

Dimerization

Dimerization and subsequent autophosphorylation are known to be essential for RTK activation. Also rPTP activity can be regulated by dimerization, although resulting in inactivation contrary to the RTKs. Dimerization has been shown to inhibit rPTPs such as CD45 and rPTPα (Weiss, Schlessinger 1998, van der Wijk, Blanchetot & den Hertog 2005). It has also been shown that rPTPα dimerizes in live cells (Tertoolen et al. 2001, Jiang et al. 1999, Jiang, den Hertog & Hunter 2000b). Dimerization could be a common regulatory mechanism for rPTPs, although it is not clear whether the process is constitutive or requires a ligand (Stoker 2005). The dimerization process has been proposed to occur according to the wedge –model, in which the membrane-proximal PTP domains (D1, figure 4) of the two monomers interact, forming the dimer (Bilwes et al. 1996). However, Barr and coworkers recently rejected this theory, replacing it by their own head-to-toe –model, in which the D1 PTP domain of each monomer interacts with the membrane-distal PTP domain D2 of the other monomer (Barr et al. 2009, Tremblay 2009).

Other ways of activation

Matrigel, the basement membrane -mimicking matrix containing laminin, collagen IV and proteoglycans, has been shown to activate the rPTP Density-enhanced phosphatase 1 (DEP-1), involving interactions between Matrigel component(s) and the extracellular domain of DEP-1 (Sorby, Sandstrom & Ostman 2001). Also, the activity of SHP1 is stimulated >1000-fold by anionic phospholipids when myelin basic protein or mitogen-activated protein kinase MAPK is used as a substrate. On the other hand, SHP1 has very low activity when tested with various protein substrates in vitro (Zhao, Shen & Fischer 1993).

2.2.4. T-cell protein tyrosine phosphatase, TCPTP

TCPTP is one of the first members identified of the PTP gene family. It belongs to the group of non-receptor type protein tyrosine phosphatases. Despite being originally cloned from a T-cell cDNA library, it is expressed ubiquitously at all stages of mammalian development and in most embryonic and adult tissues (Cool et al. 1989, Mosinger et al. 1992), although having highest expression in hematopoietic tissues (Bourdeau, Dube & Tremblay 2005). In the absence of the gene that encodes for TCPTP, mice develop normally in the uterus but die 3-5 weeks after birth, having defects in bone marrow, B-cell lymphopoiesis, erythropoiesis, and T- and B-cell functions (You-Ten et al. 1997). The bone marrow stromal cells of TCPTP -/- mice secrete high levels of interferon γ, which results in the impaired B-cell development (Bourdeau et al. 2007). The deaths of the mice at 3-5 weeks after birth could be caused by switching of hematopoietic function: during fetal and postnatal life yolk sac, fetal liver and spleen produce the hematopoietic cells, whereas this function is slowly transferred to the bone marrow while the production ceases in the fetal liver and spleen. The bone marrow of TCPTP -/- mice is unable to initiate hematopoietic function and this leads to severe anemia and an arrest of B-cell production in the marrow. These results demonstrate that TCPTP is an important regulator of the immune system (You-Ten et al. 1997).

TCPTP consists of a conserved catalytic domain and a noncatalytic C-terminal domain that varies in its size, function, and hydrophobicity due to alternative mRNA splicing. Two splice variants of TCPTP are expressed in humans, the 48 kDa form (TC48) targeted to the endoplasmic reticulum and the 45 kDa form (TC45) predominantly present in the nucleus (Tillmann et al. 1994, Lorenzen, Dadabay & Fischer 1995, Kamatkar et al. 1996, Tiganis et al. 1997). Both splice variants possess the nuclear localisation signals (NLSs), but in TC48 these signals are overridden by a hydrophobic carboxyl segment directing the protein to the ER (Lorenzen, Dadabay & Fischer 1995). TC45 is the major gene product in most human and rodent cell lines and tissues. However, the hydrophobic carboxyl segment of TC48 is very conserved in different species. This suggests that despite its lesser expression, it may serve a unique function for the TCPTP gene (Ibarra-Sanchez

et al. 2000). The noncatalytic domain of TC45 contains an autoregulatory site which modulates activity via a reversible intramolecular interaction with the catalytic domain (Hao et al. 1997). The carboxy-terminal 11 kDa segment is important in determination of TCPTP localization and regulation. This became obvious when the 37 kDa constitutively active TCPTP was created from the 48 kDa form by trypsin cleavage of the 11 kDa carboxy-terminal segment (Cool et al. 1990).

Despite the prominent localisation of TC45 in the nucleus, it is capable of translocating to the cytoplasm in response to mitogenic stimuli and cellular stress (Tiganis et al. 1998, Lam et al. 2001, Sangwan et al. 2008). Nuclear exit of TC45 is independent of the exportin CRM-1, since the hyperosmotic stress –induced nuclear exit of TCPTP was not inhibited by leptomycin B, a potent and specific nuclear export inhibitor targeting CRM-1 (Lam et al. 2001). However, Lam and coworkers found that TC45 might exit the nucleus by passive diffusion and cellular hyperosmotic stress might induce the TC45 accumulation in the cytoplasm by inhibiting nuclear import (Lam et al. 2001). Thus, nucleocytoplasmic distribution can be controlled at the level of nuclear import. Overall, globular proteins greater than 60 kDa cannot cross the nuclear pore complex (NPC) by diffusion at a significant rate (Ohno, Fornerod & Mattaj 1998), and this is why GFP-TC45 fusion protein (27+45 kDa) stays inside the nucleus (Lam et al. 2001). Importin β1 and an unidentified 116 kDa protein have been implicated in the nuclear import of TC45 (Tiganis et al. 1997).

In addition to having several RTKs as substrates, which will be discussed in the next chapter, TC45 has been shown to regulate cytokine signalling via several signal transducers and activators of transcription, STATs: STAT1 (ten Hoeve et al. 2002), STAT3 (Yamamoto et al. 2002), STAT5a&b (Aoki, Matsuda 2000), and STAT6 (Lu et al. 2007). TCPTP acts on STATs mainly within the nucleus (Bourdeau, Dube & Tremblay 2005). Simoncic and co-workers have identified two members of the Janus family of tyrosine kinases (JAKs), JAK1 and JAK3, as TCPTP targets (Simoncic et al. 2002). TCPTP has also been established as a negative regulator of Src family kinases, SFK (Shields et al. 2008).

The expression of TCPTP is cell-cycle dependent. Steady-state levels of mouse TCPTP have been shown to fluctuate in a cell-cycle dependent manner, with levels remaining low in all but late G1 stage where expression increases (Tillmann et al. 1994). Also, Ibarra-Sánchez and coworkers have shown that TCPTP plays a positive role in the progression of early G1 phase of the cell cycle through the transcription factor NF-κB (nuclear factor κB) pathway (Ibarra-Sanchez et al. 2001). The negative regulation of SFK, JAK1 and STAT3 signalling by TCPTP has recently shown to be cell cycle-dependent (Shields et al. 2008). With the present knowledge, TCPTP is not known to be overexpressed in any disease.

2.2.5. Classical PTPs as drug targets

The regulation of protein tyrosine phosphorylation by the PTPs and PTKs is highly important for the fundamental cellular processes like growth and proliferation, differentiation, survival, apoptosis, adhesion and motility (Hunter 2000). As the expression of many PTKs is upregulated in cancers, several PTK inhibitors are already on the market or in clinical trials (discussed in 2.1), whereas the PTPs are newcomers in drug development (Alonso et al. 2004). Disturbances in PTP regulation have a role in the pathogenesis of several human diseases and health concerns like cancer, diabetes, obesity and immune disorders (Andersen et al. 2004, Zhang 2001, Arena, Benvenuti & Bardelli 2005). Therefore, cellular signalling pathways regulated by tyrosine phosphorylation create very interesting opportunities for novel drug development (Ventura, Nebreda 2006). Alonso and coworkers stated that the interest of the pharmaceutical industry towards PTPs was ignited by the discovery that PTP1B acts as a negative regulator of insulin signalling (Alonso et al. 2004, Elchebly et al. 1999). Since then, several PTPs have been identified as tumor suppressors and oncogenes, and at least 30 PTPs have been implicated in cancer (Andersen et al. 2004).

2.2.5.1. Classical PTPs as oncogenes

The only PTP to be thus far recognized as a bona fide oncogene is SHP2, mutated in several types of leukemia and hyperactivated by other mechanisms in some solid tumors (Mohi, Neel 2007). SHP2 is also involved in Noonan syndrome (stated incidence 1:1000–1:2500 live births), autosomal–dominant disorder comprising short stature, anomalies of the face, and cardiac defects (Tartaglia, Gelb 2005). The molecular regulation of SHP2 activation is explained in 2.2.3.3. Regulation at the molecular level –chapter of this thesis. Briefly, many of the oncogenic mutations of SHP2 have been mapped to its autoinhibitory interface, preventing the blocking effect of N-terminal SH2 –domain on the phosphotyrosine binding site of the PTP domain. Also other classical PTPs, both rPTPs and nrPTPs, have been linked with oncogenesis, but further evidence is still needed for most of them (Ostman, Hellberg & Bohmer 2006). For example, PTPα functions as a physiological activator of the Src family kinases (SFK) (Ponniah et al. 1999), and it has been found to be overexpressed in late-stage human colon carcinoma and breast carcinoma (Tabiti et al. 1995, Ardini et al. 2000).

2.2.5.2. Classical PTPs as tumor suppressors

The PTPs have been considered as being potential tumor suppressors by nature, since they antagonize oncogenic PTK signalling (Ostman, Hellberg & Bohmer 2006). Experimental proof is found for 11 PTPs. In a colon-cancer screen six classical PTPs, namely PTPBAS, PTP36, PTPH1, RPTPρ, LAR and RPTPγ were found to be mutated in colon cancer (Wang et al. 2004). Unifying theme for the three rPTPs among these is that they all contain a FERM-domain, and for all the six classical PTPs the mutations

are found outside the PTP domain (Ostman, Hellberg & Bohmer 2006). DEP-1 has been shown to possess tumor suppressor activity when overexpressed in cultured tumor cells (Keane et al. 1996, Trapasso et al. 2000). Other classical PTPs implicated as tumor suppressors include SHP1, GLEPP1, and PTP1B (Ostman, Hellberg & Bohmer 2006).

2.2.5.3. PTP1B as a drug target

Among the PTPs, PTP1B has emerged as the best-validated drug target (Zhang, Lee 2003), being effective for type 2 diabetes and obesity. It has been shown to regulate leptin signal transduction in vivo (Zabolotny et al. 2002). The adipocyte-derived hormone leptin plays a key role in body mass regulation. Deletion of PTP1B gene in mice resulted in healthy mice, with increased insulin sensitivity and resistance to diet-induced weight gain (Elchebly et al. 1999). Introduction of PTP1B antisense oligonucleotide into mice gave similar results (Zinker et al. 2002). After these results several compounds have been developed as PTP1B inhibitors (Pei et al. 2004). Of all of those compounds, only ertiprotafib entered clinical trials before discontinuation of a phase II trial due to insufficient efficacy, together with unwanted side effects (Zhang, Lee 2003, Shrestha et al. 2007). Zhang and Zhang conclude in their review from 2007 that potent and selective PTP1B inhibitors with optimal pharmacological properties will probably emerge in the not too distant future (Zhang, Zhang 2007).

The main problems in developing inhibitors for PTP1B are selectivity and bioavailability (Zhang, Zhang 2007). Mostly the problem with selectivity has to do with the closest relative of PTP1B, TCPTP, as the two nrPTPs are structurally highly homologous. Their catalytic domain sequences are 74 % identical, and the active sites of the two proteins are identical (Johnson, Ermolieff & Jirousek 2002). It has been very demanding to develop a potent PTP1B inhibitor that would be selective also over TCPTP. For instance, YRD-motif in residues 48-50 in TCPTP is found only in TCPTP and PTP1B (Asante-Appiah et al. 2001). Asante-Appiah and co-workers have also discovered that substitution of leucine to valine at position 119 is essential in synthesizing an inhibitor that is an order or magnitude more selective over PTP1B than TCPTP (Asante-Appiah et al. 2006). Leu119 is unique to PTP1B among all the PTPs. Recently, several inhibitors have been discovered that show up to 30-fold more potential for PTP1B over TCPTP (Fang et al. 2008). Bioavailability of potential drugs can be improved by reducing charge (negatively charged inhibitors penetrate the cell membrane poorly), increasing hydrophobicity, delivery of the inhibitor to cells as a prodrug, and targeting the allosteric site (Zhang, Zhang 2007).

2.3. Integrins

Integrins are transmembrane, non-covalently associated heterodimeric cell surface receptors expressed in virtually every cell type but restricted to metazoa: no homologs

have been detected in prokaryotes, plants or fungi (Whittaker, Hynes 2002). As their name implies, the integrins integrate the extra- and intracellular environments: They regulate cell-cell, cell-ECM, and cell-pathogen contacts, participating in a wide range of biological interactions such as development, tissue repair, angiogenesis, inflammation, hemostasis, leukocyte trafficking and migration, immunological synapse formation, costimulation and phagocytosis (Springer, Wang 2004, Luo, Carman & Springer 2007). Serini and coworkers characterize the integrins as the family of receptors having the most important role in mediating cell adhesion to the ECM (Serini, Napione & Bussolino 2008).

Integrins consist of two transmembrane subunits, α and β. In mammals, 24 integrins are formed by different combinations of 18 α and 8 β subunits (Guo, Giancotti 2004) (Figure 7). Each integrin subunit consists of a large extracellular, a short transmembrane and small intracellular domain, resulting in a total of >1600 amino acids (Hehlgans, Haase & Cordes 2007). Half of the integrin α-subunits contain an inserted (I) domain of about 200 amino acids (dashed circles in Fig 7). The αI domain is the major or exclusive ligand-binding site in the integrins in which it is present. The αI domain adopts the so called Rossmann fold –conformation, in which a central β-sheet is surrounded by α-helices forming a divalent metal-ion dependent adhesion site, MIDAS (Luo, Carman & Springer 2007).

In addition to other ligands, the main ligands for the integrins are distinct members of the ECM. One group of integrins recognises the tripeptide sequence Arginine – Glycine – Aspartic acid (RGD, drawn in gray in Fig. 7) that is present in e.g. fibronectin and vitronectin in vertebrates (Hynes 2002). Another group (drawn in yellow in Fig. 7) mediates adhesion to laminins in basement membranes. Integrins α1, α2, α10, and α11 constitute the collagen receptors (green), whereas α4β1 and α9β1 recognize both ECM constituents such as fibronectin and Ig-superfamily cell surface counterreceptors such as VCAM-1. Also a group of leukocyte-specific integrins is found in vertebrates (Hynes 2002) (Figure 7).

Integrin α1β1 is a receptor for collagens and laminins, and the major collagen type IV receptor (Abair et al. 2008, Calderwood et al. 1997). The α1 subunit has the shortest cytoplasmic tail of all integrin α-subunits, with only 15 amino acids: KIGFFKRPLKKKMEK. The GFFKR –sequence is conserved in all α integrin subunits and the very basic PLKKKMEK is specific to α1 integrin (Loster et al. 2001). The α1 cytoplasmic tail is known to bind to at least the adaptor protein Shc via caveolin-1 (Wary et al. 1998). Mice lacking α1β1 are characterized by compromised tumor angiogenesis, seemingly as a result of increased levels or activity of the MMPs cleaving plasminogen to angiostatin which inhibits angiogenesis (Pozzi et al. 2000).

The cytoplasmic tails of integrins link changes in ECM composition to alteration in the intracellular actin cytoskeleton network (Berrier, Yamada 2007). These connective

points are called focal adhesions, and they comprise a complex multi-protein scaffolding and signalling unit that mediates cell adhesion and tensile forces that are important for cell motility (Zaidel-Bar et al. 2004). The cytoplasmic domains do not show intrinsic catalytic activity, and thus need associated proteins to mediate the signalling events. For example the PTKs are important in mediating the signals generated by integrin clustering. The integrins also are involved in organizing the cytoskeleton as well as activating intracellular signalling pathways such as Akt/PKB, ERK/MAPK, JUN and NFκB, promoting cell proliferation, migration and survival. Signalling of integrins proceeds predominantly via the recruitment and activation of Src-family kinases. Focal adhesion kinase FAK is recruited by β-subunits of most integrins (Guo, Giancotti 2004).
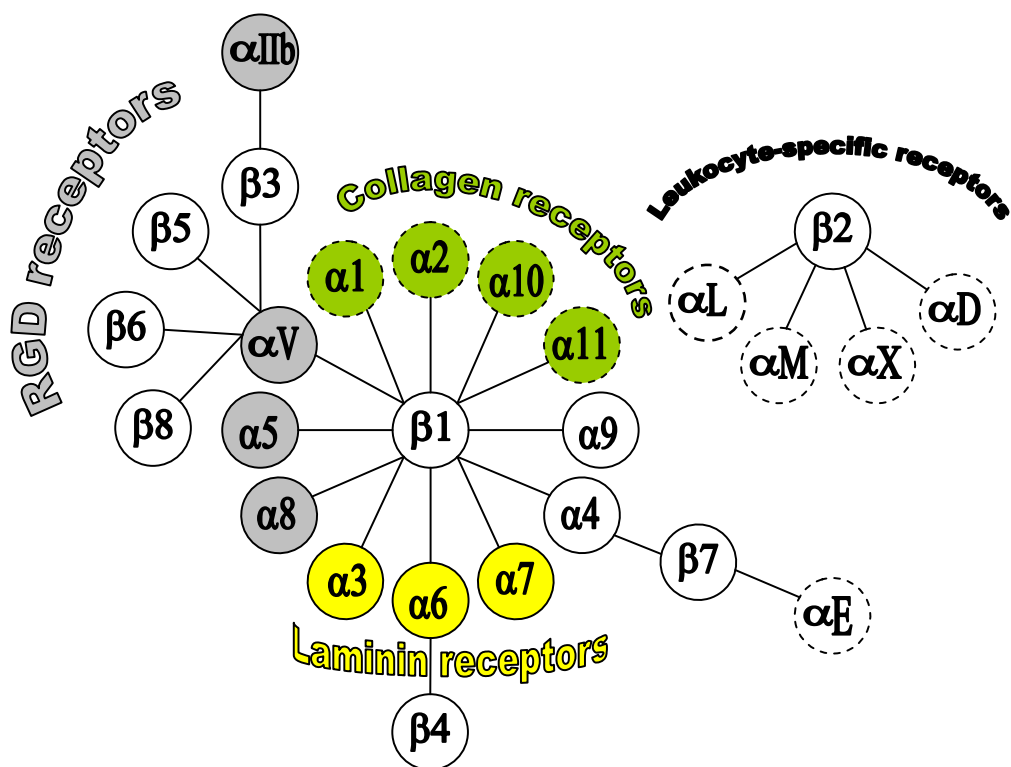
Figure 7. The integrins expressed in mammals. The collagen receptors are presented in green, laminin receptors in yellow, RGD receptors in gray, and the leukocyte-specific receptors are presented as a separate group. The α-subunits surrounded with dashed circles represent the I-domain containing integrins. Adapted from (Hynes 2002).

Integrins are involved both in outside-in signalling, in which ligand binding transduces signals from the extracellular domain to the cytoplasm, and in inside-out signalling, through which integrin adhesiveness can be dynamically regulated (Luo, Carman & Springer 2007). Changes in ECM composition are mediated to the intracellular actin cytoskeleton network by the integrins.

Integrins are also involved in angiogenesis. Broad array of integrins are expressed in vascular cells (ECs, pericytes, smooth muscle cells) including α1β1, α2β1, α4β1, α5β1, αvβ1, αvβ3, αvβ5, αvβ8, α6β1, and α6β4 (Hynes 2007).

2.4. Negative regulation of RTKs by TCPTP

In contrast to the stimulatory role of the majority of RTKs, the primary role of most PTPs is to inhibit signalling, although for example SHP2 and CD45 have also positive roles in some signalling pathways (Ostman, Bohmer 2001, Chernoff 1999). PTPs counteract both ligand-activated and ligand-independent RTK signalling. It seems that each RTK associates with and is dephosphorylated by several PTPs. The dephosphorylation can be general, terminating the receptor signalling, or target certain phosphotyrosines, modulating signalling downstream of the receptor (Persson et al. 2004a). One PTP can show both negative and positive effects on RTK signalling: SHP2 promotes signalling via EGFR but down-regulates signalling in response to PDGF (Saxton et al. 1997).

TCPTP has been found to downregulate signalling of altogether five RTKs. The results described in the Results and Discussion –sections of this thesis add one more, VEGFR2, to the list. Rather than recognizing all RTKs as substrates in vivo, TC45 shows specificity in its actions as a regulator of signal transduction. This was noted already in 1998 when Tiganis and co-workers published results on TC45 being able to recognize specific sites within selected substrates (Tiganis et al. 1998). The regulation of EGFR, PDGFRβ, Met, IR, and CSF-1R by TCPTP is discussed below (summarised in Table 1).

Table 1: RTKs negatively regulated by TCPTP.

RTK	References
EGFR	Tiganis et al. Mol. Cell. Biol. 1998; Klingler-Hoffman et al. J. Biol. Chem. 2001
PDGFRβ	Persson et al. Mol. Cell. Biol. 2004
Met/HGFR	Sangwan et al. J. Biol. Chem. 2008
IR	Galic et al. Mol. Cell. Biol. 2003
CSF1R	Simoncic et al. Mol. Cell. Biol. 2006

2.4.1. EGFR

TCPTP has been shown to dephosphorylate EGFR in response to EGF-stimulation (Tiganis et al. 1998). In fact, EGFR was the first RTK to be recognised as a TCPTP substrate 11 years ago. Tiganis and co-workers used a substrate-trapping mutant of TCPTP (TCPTP-D182A) to explore which tyrosine-phosphorylated proteins expressed in COS cells (<u>C</u>V-1 (simian) in <u>O</u>rigin, carrying the <u>S</u>V40 genetic material) would bind to it. The substrate-trapping form of a PTP has an impaired ability to catalyse a reaction, but it is still capable of tightly binding to its substrate (Flint et al. 1997). In detail, the invariant catalytic acid (Asp182 in TCPTP) that functions as a general acid catalyst to

protonate the tyrosyl leaving group in the substrate, has been mutated to alanine. As a consequence, TCPTP-D182A holds on very effectively to its substrate, which can then be detected by Western blotting. As expected, no pTyr proteins were detected in wild-type TCPTP immunoprecipitate, while both TC45 and TC48 substrate-trapping mutants bound EGFR. In response to EGF, TC45-D182A exited the nucleus where it resides in an unstimulated state, and accumulated in the cytoplasm where it bound EGFR. However, there were differences in the range of substrates bound by the different splice variants of TCPTP even though their catalytic domains are identical. This indicates that subcellular localisation is a critical factor in TCPTP substrate recognition in vivo. Importantly, the complex formation was disrupted by vanadate, a PTP inhibitor that modifies covalently the invariant, essential nucleophilic cysteine residue at the active site, revealing that the interaction occurs via the active site cysteine.

Klingler-Hoffman and coworkers discovered that TCPTP suppresses the tumorigenicity of glioblastoma cells expressing a mutant EGFR (Klingler-Hoffmann et al. 2001). Glioblastoma multiforme (GBM) is the most aggressive type of glioma. GBMs often contain mutations or amplifications of the EGFR gene. The most common mutation produces a truncated RTK known as ΔEGFR which is constitutively active and supports growth of GBM. Klingler-Hoffman and others recognised ΔEGFR as a substrate for TC45 using the substrate-trapping mutant of TC45 (TC45-D182A). TC45 was also found to inhibit the anchorage-independent growth and proliferation of ΔEGFR expressing cells, whereas TC45-D182A only inhibited cell proliferation. Importantly, neither TC45 nor TC45-D182A were able to inhibit the proliferation of the glioblastoma cells not expressing ΔEGFR (Klingler-Hoffmann et al. 2001). These results were intriguing in regards to GBM treatment, however no further studies about the effect of TCPTP on GBM have been published to date.

Other PTPs shown to have EGFR as their substrate include PTP1B (Liu, Chernoff 1997, Haj et al. 2003, Haj et al. 2002), SHP1 (Keilhack et al. 1998), LAR (Kulas, Goldstein & Mooney 1996), and PTP-σ (Suarez Pestana et al. 1999). EGFR was recognised as PTP1B substrate by immunoprecipitation in COS cells using substrate-trapping form of PTP1B (Liu, Chernoff 1997).

2.4.2. PDGFRβ

The effect of TCPTP on PDGFRβ was studied in TCPTP knockout (ko) and wild type (wt) mouse embryos as well as in fibroblasts derived from the embryos (Persson et al. 2004a). PDGFRβ was found to be hyperphosphorylated in TCPTP ko embryos, and in TCPTP ko mouse embryonic fibroblasts (MEFs) a five-fold higher ligand-induced receptor phosphorylation was observed compared to the wt cells. This effect was partly rescued by re-expressing the enzyme. TCPTP showed site-specificity in its effect on PDGFRβ. The absence of TCPTP caused largest increase, greatly exceeding the increase

detected in total tyrosine phosphorylation, in phosphorylation of Tyr1021, the binding site for PLCγ1 which has been implicated in chemotaxis. The increase in Tyr771 phosphorylation was also higher than that in total tyrosine phosphorylation, whereas the increases in Tyr751 and Tyr579 phosphorylation levels were either the same or lower than the increase in total tyrosine phosphorylation (Persson et al. 2004a). Persson and coworkers also found increased PDGFRβ phosphorylation in PTP1B knockout MEFs compared to the wt cells. However, the phospho-site distribution was different from that caused by the absence of TCPTP (Persson et al. 2004a).

On the contrary, Meng and co-workers found no difference in pTyr levels of PDGF-stimulated PDGFRβ between TCPTP siRNA and ctrl-treated Rat1-fibroblasts (Meng et al. 2004). They studied the importance of ROS production and concomitant PTP inhibition in the context of insulin-mediated signal transduction. The ROS production inactivated TCPTP transiently, leading to no difference between the TCPTP –silenced or ctrl-treated cells.

PDGFRβ associates with and is dephosphorylated by several other PTPs as well: SHP2 (Lechleider et al. 1993), SHP1 (Yu et al. 1998), LMW-PTP (Chiarugi et al. 1995), PTP1B (Haj et al. 2003), and DEP-1 (Jandt et al. 2003, Kovalenko et al. 2000).

2.4.3. Met / HGFR

Recently, TCPTP was found to negatively regulate Met (Sangwan et al. 2008). Knockdown of TCPTP in HeLa cells enhanced HGF-induced phosphorylation of Met as well as cell invasion in Matrigel. Also, substrate-trapping mutant of TCPTP was used to show the interaction with Met by immunoprecipitation. Phosphorylation of Tyr1234/1235 in the activation loop of the Met kinase domain was found to be essential for the interaction. Confocal microscopy was used to demonstrate that TC48 colocalises with Met, and that activated Met stimulates TC45 to exit the nucleus. Rescue experiment with TCPTP raised the levels of Tyr1234/1235 phosphorylation, confirming the effect to be TCPTP-dependent. It is notable here, that TCPTP is the first PTP that has been shown to regulate the phospho-sites located in the activation loop that are critical for the activity of Met.

Other PTPs reported to having the ability to dephosphorylate c-Met are the rPTP LAR (leukocyte antigen-related) (Kulas, Goldstein & Mooney 1996, Machide et al. 2006), DEP-1 (Palka, Park & Tonks 2003) and PTP1B (Sangwan et al. 2008).

2.4.4. IR

Galic and co-workers identified IR as a TCPTP substrate in 2003 (Galic et al. 2003). They found that in response to insulin stimulation, TCPTP substrate trapping mutant formed stable complexes with the IR β-subunit in HEK293 cells. The proteins also partly co-localised at the cell periphery. In TCPTP ko mouse embryonic fibroblasts the levels of

insulin-induced IR β phosphorylation were enhanced, whereas the levels were rescued by re-introducing TCPTP expression. These results suggested that TCPTP might contribute to the IR signalling regulation in vivo. Two years later Galic and others reported that TCPTP and PTP1B function in a coordinated and temporally distinct manner to regulate IR phosphorylation and signalling (Galic et al. 2005). PTP1B had been recognised as regulator of IR already earlier by others (Kenner et al. 1996). However, Galic and co-workers observed that knockdown of both TCPTP and PTP1B caused upregulation in insulin-induced PI3K/Akt signalling, while only PTP1B downregulation resulted in enhanced mitogen-activated protein kinase ERK1/2 signalling. As a conclusion they summarised that TCPTP and PTP1B show no redundancy in insulin signalling, and that the two phosphatases control both common as well as distinct insulin signalling pathways in the same cell. Regarding insulin signalling it is important to mention that insulin-stimulation also produces ROS that transiently inactivate TCPTP and thus facilitate the Tyr-phosphorylation dependent IR signalling (Galic et al. 2005).

TC45 shows site-specificity in down-regulating IR signalling. In the absence of TC45, phosphorylation at Tyr972 in IR β subunit was enhanced, while little or no effect was observed on Tyr1162 and Tyr1163 of the activation loop (Meng et al. 2004, Galic et al. 2005). Tyr972 is involved in the recruitment of IRS-1 and Shc and the activation of PI3K (Berhanu et al. 1997). This is in line with the observation that PKB/Akt is inactivated after insulin stimulation due to dephosphorylation of the IR β by TC45.

In addition to TCPTP and PTP1B mentioned above, also PTPs LAR (Kulas et al. 1995), PTPα and PTPε (Moller et al. 1995) have been shown to be involved in the negative regulation of the IR. However, Wälchli and co-workers tested 45 human PTPs, including PTPα, in a membrane binding assay using substrate-trapping mutants. They confirmed the preliminary results with secondary dephosphorylation tests and concluded that only TCPTP and PTP1B can be regarded as being physiological enzymes for the IR kinase (Walchli et al. 2000).

2.4.5. CSF-1R

TCPTP knockout mice die soon after birth because of systemic inflammatory disease (You-Ten et al. 1997, Heinonen et al. 2004). The number of macrophages in the spleen has been reported to be increased in the knockout mice, however the mechanism is not known. Simoncic and co-workers showed that TCPTP is an important regulator of CSF-1 signalling, with CSF-1R revealed as a TCPTP substrate (Simoncic et al. 2006). They were able to immunoprecipitate CSF-1R with the TCPTP substrate-trapping mutant, and show that the interaction occurs via the catalytic domain of TCPTP. To analyze which pathways downstream of the CSF-1R are affected by the loss of TCPTP, Simoncic and others stimulated TCPTP knockout and wildtype bone marrow derived macrophages (BMDM) with CSF-1 and analysed the lysates for the activation of ERK and Akt

kinases. The knockout cells displayed a dramatic increase in the level of ERK activation compared to wildtype cells, whereas no difference in Akt activation was observed.

In macrophage differentiation, the recruitment and phosphorylation of a Gab2/SHP2 complex to the CSF-1R is a critical signalling event. Gab2, the GRB2-associated binding protein 2, is the principal PI3K activator. It has been shown that the expression of a mutant Gab2 unable to bind SHP2 inhibits CSF-1 –induced macrophage differentiation (Liu et al. 2001). Also, ERK activation downstream of several other RTKs requires that SHP2 is phosphorylated and activated (Gu, Neel 2003, Liu, Rohrschneider 2002, Neel, Gu & Pao 2003). Therefore, Simoncic and co-workers studied the tyrosine phosphorylation of Gab2 and SHP2 after stimulation with CSF-1 in TCPTP knockout and wildtype BMDMs. They found that after CSF-1 stimulus, TCPTP knockout BMDMs show increased tyrosine phosphorylation of Gab2 and SHP2 (Simoncic et al. 2006). To conclude, TCPTP was recognised as a novel regulator of CSF-1 signalling and mononuclear phagocyte development.

3.   AIMS OF THE STUDY

Cells control their growth, proliferation and differentiation by phosphorylation of key proteins on specific tyrosine residues. This balance is maintained by the contrary actions of protein tyrosine kinases and protein tyrosine phosphatases. The general aim of this research was to elucidate the role of T-cell protein tyrosine phosphatase, which we found by Yeast-two-hybrid screening using α1 integrin cytoplasmic tail as the bait, in the regulation of the receptor tyrosine kinases EGFR and VEGFR2. In addition, the activation mechanism of TCPTP by the α1 integrin was studied.

The specific objectives of this study were

1.   To investigate whether α1 integrin –induced TCPTP activity would cause EGFR downregulation

2.   To elucidate whether also VEGFR2 would be a TCPTP target

3.   To search for small molecule activators for TCPTP

4.   To determine whether the most potent small molecule TCPTP activator, Spermidine, would be capable of affecting EGFR and PDGFRβ signalling

5.   To evaluate the activation mechanism of TCPTP by α1 integrin cytoplasmic tail and Spermidine

4. MATERIALS AND METHODS

More detailed information on methods and reagents is available in the original publications (I-III).

Expression vectors

The following expression vectors were generated:

(used in I): pGBKT7-α1, pGEX4T1-α1cyt, pGEX4T1-α2cyt, pGEX4T1-α10cyt, pGEX4T1-α11cyt, pGEX4T1-SHP2, pCG-TC45, pGEX-6P1
(used in II, III): pcDNA3.1(+)-TC37
(used in III): pGEX-TC45, pGEX-TC45-E8,11A, pGEX-TC45-E24,28A, pGEX-TC45-E8,11,24,28A

TCPTP shRNA construct (used in I):

pRNA-U6.1/Neo-TCPTP

Expression vectors with the following inserts were kindly provided:

pcDNA3.1/zeo-α1 (A. Pozzi, Vanderbilt University; used in I)
pCG-TC45, pCG-TC45-D182A, pCG (T. Tiganis, Monash University, Australia, used in II)
pCDNA3.1-VEGFR2 (L. Claesson-Welsh, Uppsala University, Sweden, used in II)

Cell lines

Cell line	Species	Cell type / organ of origin	Used in
HeLa	human	cervical squamous epithelial	I, II, III
HEK-293	human	embryonic kidney epithelial	II
PC-3	human	prostate gland epithelial	I
α1 -/- MEFs	mouse	embryonic fibroblasts	I
α1 +/+ MEFs	mouse	embryonic fibroblasts	I
B104-1-1	mouse	embryonic fibroblasts	I
Saos-2	human	osteogenic sarcoma	I
HT1080	human	fibrosarcoma	I
HUVEC	human	umbilical vein endothelial	II, III
PAE-Flt1	porcine	aortic endothelial	II
EFM7+/+	mouse	embryonic fibroblasts, TCPTP wt	III
EFM7-/-	mouse	embryonic fibroblasts, TCPTP ko	III

The following stable cell lines were generated (used in I):

Saos-2-α2α1, Saos-2-α2α5
(Saos: a non-transformed cell line derived from the primary osteosarcoma of an 11-year old Caucasian girl)

Primary antibodies

Antigen	Antibody and/or supplier	Used in
TCPTP	CF4, mouse ab, Calbiochem Oncogene	I, II, III
TCPTP	3E2, mouse ab, Prof. Tremblay	II, III
TCPTP	TCPTP polyclonal, Pharmingen	II
α1 integrin	mAb 1973, clone FB12, Chemicon	I, II
α1 integrin	Ab 1934, Chemicon	II
α2 integrin	MCA2052, Serotec	I
α5 integrin	Chemicon	I
α6 integrin	Chemicon	I
β1 integrin	MB1.2, Chemicon	I
β1 integrin	mAb 2252, Chemicon	II
β3 integrin	Serotec	II
SHP2	Santa Cruz	I
α-tubulin	Santa Cruz	I
α-tubulin	Hybridoma bank	II, III
EGFR	Rabbit mAb, Cell Signalling Tech.	I
phospho-EGFR	Rabbit mAb pY1068, Cell Signalling Tech.	I, III
phospho-EGFR	Rabbit mAb pY845, Cell Signalling Tech.	I
phospho-EGFR	Rabbit mAb pY992, Cell Signalling Tech.	I
phospho-Met	Rabbit mAb pY1349, Cell Signalling Tech.	I
phospho-Met	Rabbit mAb pY1234/1235, Cell Signalling Tech.	I
VEGFR2	Rabbit mAb 55B11, Cell Signalling Tech.	II
VEGFR1	Rabbit mAb, Cell Signalling Tech.	II
phospho-VEGFR2	Rabbit mAb pY996, Cell Signalling Tech.	II
phospho-VEGFR2	Rabbit mAb pY1175, Cell Signalling Tech.	II
phospho-VEGFR2	Rabbit mAb pY1214, Cell Signalling Tech.	II
phospho-VEGFR2	Rabbit mAb pY1054/1059, Sigma	II
vinculin	Sigma	II
phosphotyrosine	PY20, Transduction Laboratories	II
phospho-PDGFRβ	pY1021, Santa Cruz	III

Growth factors

EGF (Sigma) was used to activate EGFR (used in I, III), VEGF165 (Peprotech) was used to activate VEGFR2 (used in II, III), PDGF-BB (Cell Signalling Technology) was used to activate PDGFRβ (used in III).

Peptides

TAT-peptides (used in I, II, III), Genemed Synthesis / Innovagen:
α1-TAT: FITC-N-YGRKKRRQRRRWKLGFFKRPLKKKMEK-C
α1-B-N-TAT: YGRKKRRQRRRRPLKKKMEKRPLKKKMEK-C
Scr-TAT: FITC-N-YGRKKRRQRRRLKGWRFKLKPKFKEMK-C

Synthetic integrin cytoplasmic tail peptides (used in I, II, III):

α1: N-RPLKKKMEKRPLKKKMEK-C,

α2: N-KLGFFKRKYEKMTKNPDEIDETTELSS-C, both gifts from J. Heino.

Chemicals

Chemical	Application	Supplier	Used in
DiFMUP	phosphatase assay	Molecular Probes	I, II
Ser/Thr phosphatase inhibitor cocktail	phosphatase assay	Sigma	I
Mowiol	immunofluorescence	Calbiochem	I
1,4-diazadicyclo-2.2.2.-octane (DABCO)	immunofluorescence	Sigma	I
Fluorescein diphosphate	immunofluorescence	Molecular Probes	I
WST-1	proliferation assay	Roche	I
Sodium orthovanadate	immunoprecipitation	Sigma	II
PPase inhibitor cocktails I and II	phosphatase assay	Sigma	II
EZ-Link Sulfo-NHS-SS-Biotin	internalization assay	Pierce	II
Sodium 2-mercaptoethanesulfonate (MesNa)	internalization assay	Fluka	II
Cell Titer Blue	proliferation assay	Promega	III
Spermidine trihydrochloride	various assays	Sigma	III
Mitoxantrone	various assays	Sigma	III
Ruthenium red	various assays	Sigma	III
MDL-26,630-trihydrochloride	various assays	Sigma	III
Methyl cellulose	spheroid assay	Sigma	III

Libraries

Small molecule library	Compounds	Supplier	Used in
Spectrum Microsource	2000	Microsource Discovery Systems	III
LOPAC1280	1280	Sigma	III
ChemDiv	25000	ChemDiv Incorporated	III
ChemBridge	30000	ChemBridge Corporation	III
Tripos	6000	Tripos International	III

Library for Yeast-two-hybrid screen (used in I):

Mouse E17 cDNA library, Clontech.

siRNAs

Target	Sequence (sense) or name	Supplier	Used in
TCPTP	5-GGCACAAAGGAGUUACAUCTT-3	Ambion	I
TCPTP	5-GGAGUUACAUCUUAACACATT-3	Ambion	I, II, III
SHP2	5-GGAGUUGAUGGCAGUUUUUTT-3	Ambion	I
SHP2	5-GGCCUAGUAAAAGUAACCCTT-3	Ambion	I
scr ctrl	Non-specific control duplex II	Dharmacon	I
scr ctrl	All Stars negative control	Qiagen	II, III

Methodology

Method	Used in
Cell culture	I, II, III
Yeast-two-hybrid screen	I
Protein-protein interaction assay	I
Immunoprecipitation	I, II
Western blot analysis	I, II, III
Phosphatase assay	I, II, III
Transfection	I, II, III
Generation of stable cell lines	I
Adhesion assay	I
Bead binding assay	I
Immunofluorescence	I, II
Soft agar assay	I
FACS analysis	II
Kinase assay	II
Proliferation assay	I, II, III
Tube formation assay	II
Sprouting angiogenesis assay	II, III
VEGFR2 internalization assay	II
Time-lapse chemokinesis assay	II
Chemotaxis assay	II
Site-directed mutagenesis	III
High-throughput screening	III
Surface plasmon resonance assay	III
Pull-down assay	III
Statistical analysis	I, II, III

Microscopes

Axiovert 200M inverted fluorescence microscope, Carl Zeiss Microimaging (used in I, II)

Confocal laser scanning microscope Axioplan 2 with LSM 510, equipped with x63/1.4 Plan-Apochromat oil immersion objectives, Carl Zeiss Microimaging (used in I, II)

Animals

Mice: Female athymic Nude-nu, Harlan Scandinavia, Allerod, Denmark, age 6-8 weeks at the start of the experiments.

5.   RESULTS

5.1.   EGFR signalling is negatively regulated through integrin-α1β1-mediated activation of TCPTP (I)

5.1.1. The integrin α1 cytoplasmic tail associates with TCPTP and activates it

To search for binding partners of a collagen-binding integrin α1, we performed a Yeast-two-hybrid screen. After recognising TCPTP as a putative binding partner for integrin α1 cytoplasmic tail, we verified this interaction in cells. Immunofluorescence results showed that endogenous α1 integrin and TCPTP co-localize in peripheral areas of the membrane in PC3 and HeLa cells adhering to collagen, whereas on fibronectin or poly-L-lysine endogenous TCPTP was diffusely in the cytoplasm, or in both the cytoplasm and the nucleus, respectively (I, Fig. 1a & b). Cells bind to collagen mainly via integrin α1, to fibronectin via integrins α5 and α6, whereas the binding to poly-L-lysine is non-integrin-dependent. Reciprocal immunoprecipitations in HeLa cells with α1 and TCPTP antibodies were used to show that the endogenous proteins associate with each other after cell binding to collagen or serum stimulus (I, Fig. 1c & d) and the association is specific to α1 integrin (I, Fig. 1e).

Next we studied whether α1 integrin cytoplasmic tail (α1-cyt) would be able to activate TCPTP. Previous in vitro studies had suggested that the catalytic activity of TCPTP is regulated by an intramolecular inhibition involving a carboxy-terminal segment of the 45 kDa form of TCPTP (Hao et al. 1997). We hypothesized that α1-cyt could activate TCPTP by competing with and overriding the autoinhibition. As expected, cell adhesion to collagen as well as treatment with a synthetic α1-cyt induced catalytic activity of TCPTP significantly and specifically in phosphatase assays (I, Figs S2a, 2a & b). A 37 kDa mutant lacking the alleged regulatory C-terminal segment was not activated by α1-cyt, in line with our hypothesis (I, Fig. 2b). Further experiments with GST-TCPTP deletion mutants suggested that the α1-cyt associates with the N-terminal part of TCPTP (I, Figs 2c & d). This association would then lead to TCPTP activation, by preventing the proposed autoregulatory C-terminal segment from interacting with the N-terminal part of the protein.

5.1.2. Integrin α1β1 ligation attenuates EGFR phosphorylation through activation of TCPTP

It had been shown that TCPTP is a substrate for EGFR and that its overexpression downregulates EGFR phosphorylation and signalling (Tiganis, Kemp & Tonks 1999). We decided to study how cell adhesion to matrix and subsequent TCPTP activation would affect EGFR phosphorylation. A striking downregulation of EGFR phosphorylation was

observed in HeLa cells adhering to collagen after EGF stimulation, as compared to cells on plastic (I, Figs 3a & b). The effect was strongest already after 5 min. stimulus with EGF (I, Fig. 3b), and this timepoint was hence used hereafter. To exclude the possibility that the attenuation in EGFR phosphorylation was due to influence by other integrins or simply detachment and replating, we plated HeLa on fibronectin as well as on collagen and otherwise performed the assay similarly. Cells adhered well on both surfaces (I, Fig. S3a), but EGF-induced phosphorylation was remarkably lower in cells on collagen (I, Fig. 3c).

TCPTP knockdown in HeLa and mouse fibroblasts devoid of α1 integrin were used to confirm that the effect seen on EGFR phosphorylation was specifically due to the influence of TCPTP and α1-cyt. EGF-induced EGFR phosphorylation on cells plated on collagen was indeed clearly stronger in cells in which TCPTP had been silenced. SHP2, another non-receptor PTP, was silenced as a control and this had no effect on collagen-induced attenuation of EGFR phosphorylation (I, Fig. 3d). In α1 -/- fibroblasts, using two separate isolates, no difference in EGF-induced EGFR phosphorylation was observed between cells plated on collagen or fibronectin, indicating that α1 integrin is responsible for the effect seen again in the wild type cells (I, Fig. 3e). Finally, to further support the role of integrin α1 in TCPTP-mediated attenuation of EGFR signalling, we used α1β1-null HT1080 cells in which adhesion to collagen had no effect on EGFR phosphorylation (I, Fig. S3b), HeLa cells where cytosolic PTP activity was increased by 1,5-fold as a result of integrin α1-subunit clustering by antibody (I, Figs S2b & S3d), and the α1 -/- fibroblasts in which TCPTP was not recruited to the focal complexes with α1 integrin as in the wt cells (I, Fig. S3e). These findings comprehensively show that α1 integrin is responsible for TCPTP activation, and this interplay results in subsequent downregulation of EGFR phosphorylation.

5.1.3. α1 cytoplasmic tail peptide induces phosphatase activity in vivo and inhibits anchorage-independent and EGF-induced cell growth

In order to show that α1-cyt is able to activate TCPTP also in vivo, we performed several experiments. A cell-permeable TAT-peptide was used to transport α1-cyt or scrambled peptide into the cells in proliferation and tumorigenicity assays. Proliferation of non-adherent HeLa (I, Fig. 4a) and B104-1-1 cells (I, Fig. 4c) was detected in the presence or absence of 200 nM α1-TAT or scrTAT and either 50 ng/ml EGF (HeLa) or 10 % fetal bovine serum (FBS) (B104-1-1) for 0, 24, and 48 h. α1-TAT peptide was capable of significantly suppressing the proliferation of both cell types in the presence of the mitogens especially at the 48 h timepoint using the WST-1 reagent (I, Fig. 4a & c). Also, ability of HeLa to form colonies in soft agar was tested in the presence or absence of the 200 nM TAT-peptides and 10 % FBS. Colonies formed by cells treated with α1-TAT were significantly smaller as compared to both scrTAT-treated and non-treated cells (I,

Fig. 4d). Furthermore, no large colonies had formed in the presence of α1-TAT (I, Fig. 4d). We also tested the ability of the TAT peptides to regulate EGFR signalling. α1-TAT significantly downregulated EGFR phosphorylation at tyrosine 1068 after stimulation with 50 ng/ml EGF (I, Fig. S4c).

To investigate the TCPTP-specificity of the α1-TAT effects, we generated stably transfected cells with TCPTP knockdown (HeLapRNAU6.1TCPTP). Short hairpin-RNA-mediated downregulation of TCPTP rendered HeLa cells less responsive to α1-TAT as compared to ctrl shRNA-treated cells (I, Fig. 4b). In addition, these TCPTP-silenced and control cells plated on collagen were tested for phosphatase activity after microinjecting fluorescein disphosphate (FDP) that becomes fluorescent upon dephosphorylation, and α1 or α2 integrin cytoplasmic tail peptides. Phosphatase activity induced by α1 peptide was remarkably lower in TCPTP knockdown cells than in control cells (I, Fig. S4b). These results demonstrate at cellular level that the effect of α1-TAT on cell proliferation most likely proceeds via TCPTP.

5.2. TCPTP controls VEGFR2 signalling (II)

5.2.1. Human endothelial cells express TCPTP in vitro and in vivo

Inspired by the findings at cellular level reported in the first publication (see above), we wanted to investigate whether α1TAT peptide could inhibit cell proliferation in human xenografts in vivo. Thus, we performed several experiments in vivo in athymic female nude mice (Harlan), aged 6-8 weeks at the start of the experiment. α1-null HT1080 cells ($5x10^6$), pre-treated with α1TAT, scrTAT, or not pre-treated, were injected in PBS subcutaneously into one flank of each mouse. Starting a day after the injections of the cancer cells, the injection sites were treated 3 times a week for 4 weeks with 10μM α1TAT, scrTAT, or PBS as a control, increasing the volume as the tumor volume grew. Injection volume of the peptides was 10 μl / 0,1 cm^3 tumor, however the minimum volume was 20 μl and the maximum 250 μl per tumor. The preliminary experiment with only 3 mice per group indicated that α1TAT makes the tumors necrotic, and restricts the growth of the tumors both after day 23 and in total (post mortem weights of the tumors) (Fig. 8 and not shown). Also the amount of capillaries formed in tumors of mice treated with α1TAT peptide was dramatically smaller compared to those of scrTAT or PBS-treated mice (not shown).
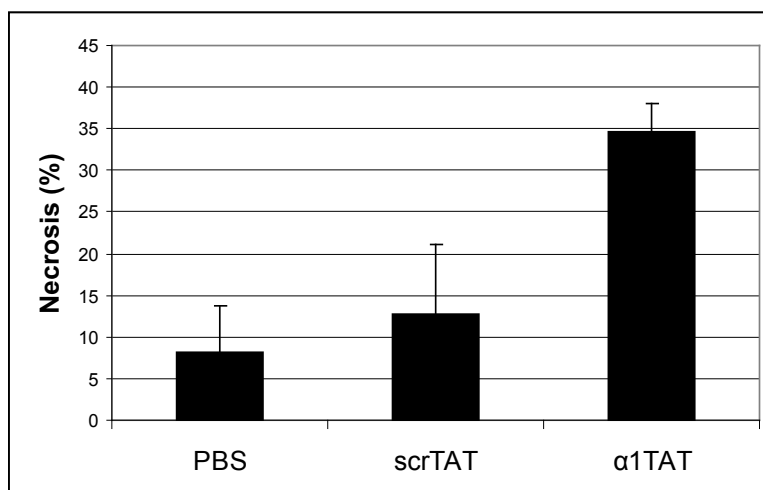
Figure 8. Necrosis of HT1080-tumors in athymic nude mice. Masson Trichrome staining was performed to assess necrosis from nude mice injected with HT1080 cells and treated as indicated. Results from the pilot experiment with only 3 mice per group. Shown is the percentage of necrosis + SEM.

However, repeats of the experiment with larger numbers of mice (n=10 per group) did not produce as promising results. Also in those experiments α1TAT was able to restrict the growth of the tumors but due to a large variation in the treatment responses there was no statistically significant difference between α1TAT and scrTAT or PBS –treated mice, only a trend. None of the mouse test results described above have been published because of the lack of statistical significance.

Still, excited about the finding that we were able to see a dramatic decrease in the amount of blood vessels formed and an increase in the amount of necrosis in the pilot mouse experiment, we decided to explore if α1 integrin – TCPTP interplay would have an effect also on the main RTK involved in angiogenesis, VEGFR2. For this to be conceivable, endothelial cells would need to express both α1 integrin and TCPTP. We studied TCPTP expression in early passage human umbilical vein endothelial cells (HUVEC), peripheral blood mononuclear cells (PBMC), and tissue samples of human umbilical cord and inflamed tonsils. TCPTP was detected in all of these cells and tissues (II, Figs 1a - d, S1b). Notably, immunocytochemistry revealed that TCPTP is present both at the membrane and in the cytoplasm of HUVECs (II, Fig. 1c). In frozen sections of human umbilical cords, TCPTP, α1 integrin and VEGFR2 were all expressed in the endothelial layer of the veins (II, Fig. 1d). α1β1 integrin was detected in HUVECs (II, Fig. 1a), and cell surface expression of α1 integrin on HUVECs was shown by FACS analyses (II, Fig. S1a). These results indicate that TCPTP has a role in vascular biology.

5.2.2. TCPTP activity controls VEGF-dependent responses in endothelial cells

In order to study whether TCPTP plays a role in VEGF-dependent responses, we induced TCPTP activity or silenced its expression in HUVECs with methods described above, and measured changes in proliferation and migration. TCPTP downregulation increased VEGF-induced proliferation significantly (28 ± 6 %, $p < 0,05$) compared to scr siRNA –transfected or nontransfected cells (II, Fig. 2a). To detect the effect of activated TCPTP on migration, we used a constitutively active 37 kDa mutant of TCPTP (TC37). It is a truncation mutant of TC45, in which the C-terminal noncatalytic segment has been removed. According to previous studies, this segment contains an autoregulatory site which binds to the catalytic site, keeping TC45 in an autoinhibited state unless activated by mitogens or integrin $\alpha1\beta1$ (Hao et al. 1997) (I). Chemotaxis towards VEGF of HUVECs transfected with the TC37 or empty vector was measured in a Transwell assay. Expression of TC37 practically hindered the migration towards the chemoattractant (II, Fig. 2b). As a conclusion, VEGF-dependent responses, proliferation and migration, are promoted by TCPTP downregulation, and suppressed by its activation. These results prompted us to investigate the probable interaction between TCPTP and VEGFR2.

5.2.3. TCPTP binds to VEGFR2, dephosphorylates it site-specifically, and controls its activity

A widely used method for searching PTP substrates is the substrate-trapping technique (Flint et al. 1997). Complexes formed by enzymes and substrates are very temporary and hence difficult to detect. This can be overcome by using a substrate-trapping mutant in which the ability to bind substrates is retained, but catalytic activity is impaired, and as the result the mutant holds on to the substrate tightly. We performed reciprocal immunoprecipitations in HEK293 cells with VEGFR2 and TCPTP antibodies to detect the interaction between the enzyme TCPTP and the potential substrate VEGFR2. HEK293 cells were transfected with VEGFR2 and the substrate-trapping mutant of TCPTP (TC45-D182A), and stimulated with 100 ng/ml VEGF for 5 minutes. The lysates were immunoprecipitated with antibody against TCPTP or IgG for control. TCPTP bound to VEGFR2 efficiently (II, Fig. 3a). Reciprocally TCPTP was successfully immunoprecipitated with VEGFR2 in a similar experiment (II, Fig. 3b). Notably, use of sodium orthovanadate (Na_3VO_4) prevented the formation of the complex, suggesting that the binding occurs through the active site cysteine of TCPTP.

Next we wanted to find out if in addition to binding to VEGFR2, TCPTP would also be able to dephosphorylate it. Indeed, purified, recombinant TCPTP decreased phosphorylation of immunoprecipitated VEGFR2 by 68 ± 11 % in the presence of VEGF when total phosphotyrosine levels were detected (II, Fig. 4a). To investigate which specific phosphotyrosines are affected by TCPTP, similar experiment was used. There are several important autophosphorylated tyrosine residues on the VEGFR2 dimer. Of

those, we looked at five different ones. Tyrosine 1214 has been linked to p38 signalling cascade via Src family kinase member Fyn and SH2 domain-containing adapter molecule Nck (Lamalice, Houle & Huot 2006), Tyr1175 has been shown to bind to Shb and PLCγ (Holmqvist et al. 2004, Cunningham et al. 1997), while Tyr1054 and 1059 located in the activation loop of the kinase domain are critical for the catalytic activity of VEGFR2 (Kendall et al. 1999). TCPTP was able to markedly dephosphorylate tyrosines at positions 1214, 1054/1059, and 996, located in the kinase insert domain but target of which is currently unclear (II, Fig. 4b). Tyr1175 was not a target for TCPTP.

Growth factor receptors are internalized after activation with specific ligands and subsequent phosphorylation (Lampugnani et al. 2006). Studying internalisation of VEGFR2 would thus be another means by which to study receptor phosphorylation. We used two methods to investigate this. First, immunofluorescence was used to study internalisation of VEGFR2. Subconfluent HUVECs were transfected with constitutively active TCPTP (TC37) or with vector control (pCG) and treated with 50 ng/ml VEGF for 5 min. or left unstimulated. Cells were fixed, permeabilised, and stained for VEGFR2 (red) and nuclei (blue). As a result, there are clear VEGFR2 –containing vesicles in control-transfected cells, whereas TC37 has inhibited the internalisation efficiently (II, Fig. 4c). The number of VEGFR2-positive vesicles was quantitated using image analysis (mean ± SEM, *** p<0,005). Degree of VEGFR2 internalisation was analysed also by a biotinylation assay. Cell surface proteins of TC45, TC37 or vector-transfected cells in the presence or absence of 100 ng/ml VEGF were biotinylated and internalisation was allowed to proceed for 15 min. Lysates were analysed for biotin, i.e. internalised VEGFR2, and VEGFR2 as a loading control. Biotinylated receptors remaining on the cell surface are cleaved using a reducing agent, thus only the internalised ones are detected in the immunoblot (Ivaska et al. 2002).

Based on these results we conclude that TCPTP binds to VEGFR2, is able to dephosphorylate it site-specifically and controls internalization of the receptor.

5.2.4. Adhesion of endothelial cells to collagen activates TCPTP

TCPTP and integrin α1β1 colocalise in HUVECs adhering to collagen IV (II, Fig. 5a), which is the primary substrate of α1β1 integrin. On gelatin, to which cells bind mainly via integrin αvβ3, TCPTP resides mostly in the nucleus and does not colocalize with integrin α1β1. Binding to collagen IV also activates TCPTP, as detected by measuring phosphatase activity from cells grown on collagen and immunoprecipitated with antibody against TCPTP (II, Fig. 5b). HUVECs display basal TCPTP activity also on gelatin, but this activity is increased by 47 ± 1 % (p < 0,05) on collagen IV (II, Fig. 5b). As a conclusion, α1-mediated adhesion to collagen IV activates TCPTP.

5.2.5. The cytoplasmic domain of α1 integrin activates TCPTP and inhibits VEGFR2 activity

To determine how α1 integrin – TAT –peptide (α1-TAT) which we used already in the first publication would function as a TCPTP activator and affect VEGFR2 signalling, we performed three different assays. First, we used immunoblotting to determine the effect of the TAT-peptides on VEGFR2 phosphorylation at sites Tyr1214 and Tyr1175. Serum-starved, subconfluent HUVECs were treated for 1h with α1- or scrTAT peptides or left untreated, and stimulated or not with 100 ng/ml VEGF for 15 min. Lysates were resolved on SDS-PAGE and blotted for VEGFR2 Tyr1214 or Tyr1175 and tubulin and VEGFR2 as a control. α1-TAT had a clear, downregulatory effect on Tyr1214, whereas Tyr1175 was not affected, similarly as in II, Fig 4b. Second, the TAT-peptides were analysed for influence on VEGFR2 kinase activity. In the presence of VEGF, α1-TAT abolished the kinase activity as compared to scrTAT and untreated cells (II, Fig. 5d). Third, we incubated recombinant, purified TCPTP with α1 or α2 cytoplasmic tail peptides, the TAT peptides, or with no peptide, and measured phosphatase activity (II, Fig. 5e). α1-TAT induced TCPTP activity more than twofold, as compared to any of the controls D-scrTAT, α2-peptide or the sample treated with no peptide. To conclude, α1-TAT peptide can be used as a specific tool to dephosphorylate VEGFR2 and inactivate it in cells.

5.2.6. TCPTP activation by the cytoplasmic tail of integrin α1 inhibits endothelial proliferation, migration, morphological changes, and sprouting angiogenesis

VEGFR2 is the main mediator of VEGF-stimulated endothelial cell migration, proliferation, survival, and enhanced vascular permeability (Roskoski 2007). Having now shown that TCPTP activation has a negative effect on VEGFR2 signalling, we wanted to study how α1-TAT as a TCPTP activator would influence the proliferation, migration, and capillary-like formation of endothelial cells. In a fibrin-gel angiogenesis assay with HUVECs we show that in the presence of VEGF, α1-TAT is able to reduce morphological changes resembling capillary formation of endothelial cells, by 60 % when compared to non-treated cells (p < 0,01) and by 40 % when compared to scrTAT –treated cells (II, Fig. 6a). A sprouting angiogenesis assay was also utilised to study three-dimensional cultures of spheroids formed by HUVECs (II, Figs 7a&b) and porcine aortic endothelial cells (PAE, II, Fig 7c). The spheroids were cultured inside collagen gel and their ability to form sprouts, both the number of sprouts and cumulative sprout length, in the presence or absence of the TAT-peptides and VEGF was followed for 24h (II, Figs 7a-c). In all of these assays, α1-TAT was able to significantly reduce the length of the sprouts, as well as suppress the number of sprouts formed in the presence of VEGF.

Effect of the TAT-peptides and VEGF on the migration of HUVECs was investigated by treating the cells growing on gelatin with or without the TAT-peptides and VEGF for 1h. The cumulative migration distance was measured and plotted as a function of

time (II, Fig. 6b). α1-TAT treated cells migrated significantly less compared to cells treated with scrTAT or non-treated cells (II, Fig. 6b). This same effect was seen also in Transwell chemotaxis assays where cells migrate towards a chemoattractant, in this case VEGF, with different speed depending on treatment. In non-transfected HUVECs, α1-TAT markedly reduced cell migration towards VEGF (II, Fig. 6c), while in TCPTP siRNA-treated cells the peptide had no effect (II, Fig. 6d).

These results clearly show that α1 integrin cytoplasmic tail has an inhibitory effect on endothelial cell proliferation, migration, capillary-like tube formation, and VEGF-driven sprouting angiogenesis via TCPTP.

5.3.   A novel TCPTP activator attenuates EGFR and PDGFRβ signalling (III)

5.3.1. A small molecule screen revealed new TCPTP activators

We performed a small molecule high-throughput screen to identify small molecular compounds capable of activating TC45. Five small molecule libraries, Spectrum Multisource with 2000 biologically active and structurally diverse compounds, LOPAC1280 with 1280 pharmaceutically active compounds, Tripos with 6000 compounds, ChemDiv with 25000 compounds and ChemBridge with 30000 compounds were screened in an in vitro phosphatase assay. The compounds were applied on 384-well plates. Compound interference was taken into account by measuring background fluorescence from plates which contained the compounds and the reaction mix, but no TC45. TC45 activity assay was initiated by adding purified phosphatase to the wells and allowing dephosphorylation of phosphatase substrate DiFMUP to proceed for 10 minutes. The dephosphorylation reaction was stopped with urea and the fluorescence measured using a multilabel plate reader (III, Fig. 1a). Due to the autofluorescence of some compounds, the true hits were revealed by comparing the background and assay fluorescence values. After this removal of false positives, 213 putative TC45 activators were found in the primary screen (III, Table 1). A secondary screen confirmed that six of the compounds activated TC45 in a concentration-dependent manner (III, Fig. 2a). The molecules capable of activating TC45 in vitro were spermidine trihydrochloride (spermidine), mitoxantrone, ruthenium red, and MDL-26,630-trihydrochloride (MDL), the chemical structures of which are shown in III, Fig. 1c. All four compounds were shown to activate TC45 highly significantly, at least 1,7 –fold (III, Fig. 2b), mitoxantrone being the most potent.

5.3.2. Spermidine inhibits serum-induced cell proliferation in a TC45-dependent manner

Even though the identified compounds activate TC45 in vitro in a manner similar to α1 cytoplasmic tail, it is likely that some of the compounds bind other targets in the cell as

well. To study the specificity of these compounds to TC45, we tested the four compounds for their effect on cell proliferation in TC45 knockout and wt mouse embryonic fibroblasts. The cells were incubated with the compounds at indicated concentrations for 72h and proliferation was measured. Strikingly, Spermidine displayed specificity towards TC45, since TC45 knockout cells were 43-fold more resistant to the compound than wt cells, suggesting that the presence of TCPTP makes the wt cells more sensitive to the drug regarding proliferation (III, Fig. 3). The other three compounds did not show specificity towards TCPTP.

5.3.3. Spermidine regulates EGFR and PDGFRβ signalling via TC45

TC45 has been shown to negatively and site-selectively regulate PDGFRβ phosphorylation (Persson et al. 2004a). To investigate whether Spermidine would be able to attenuate PDGFRβ signalling in a TC45-dependent manner, we used TC45 knockout and wt mouse embryonic fibroblasts. We studied the phosphorylation levels of PDGFRβ Tyr 1021 in Spermidine-treated cells in the presence or absence of PDGF-BB, after overnight starvation. In line with previous data (Persson et al. 2004a), we observed that PDGF-BB induced $45 \pm 3{,}8$ % higher phosphorylation of PDGFRβ in TC45 ko. cells compared to wt cells. We found that Spermidine was able to downregulate PDGF-induced PDGFRβ phosphorylation by $42 \pm 6{,}3$ % in TC45 wt cells whereas no inhibition was detected in TC45 negative cells (III, Fig. 4A and its quantitations in B and C).

Previously we have shown that a collagen-binding integrin α1β1 negatively regulates EGFR phosphorylation via coupling to TC45 in malignant epithelial cells (I). This was the first demonstration of an inhibitory role for ECM in signalling by RTKs. Since our results with Spermidine implied that it would function in a similar manner with integrin α1 cytoplasmic tail, we tested if Spermidine would have an effect on EGFR phosphorylation in HeLa cells. Serum-starved cells were incubated in the presence or absence of 10 μM Spermidine for 1h, after which they were stimulated or not with 50ng/ml EGF for 5 or 15 minutes. Cells were lysed and the lysates run on gel and blotted for tyrosine 1068 phosphorylation of EGFR, and tubulin as a control. Spermidine was sufficient to significantly attenuate EGFR phosphorylation in cells (Fig. 4D and its quantitation in E), in a manner similar to TAT-α1-cyt peptide. These data indicate that Spermidine-induced activation of TC45 results in negative regulation of PDGFRβ and EGFR in cells.

5.3.4. Endothelial cell sprouting is inhibited by Spermidine

Our results showed that VEGFR2 is under the negative regulation of TC45 and α1 integrin in endothelial cells (II). We demonstrate that α1-TAT peptide significantly reduced the length of the VEGF-induced sprouts in three-dimensional cultures of HUVECs in a TC45 dependent manner (II). These results showed that activation of TC45 through

the cytoplasmic domain of integrin α1 leads to inhibition of VEGF-driven sprouting angiogenesis in vitro. Here we tested the effect of Spermidine on VEGF-induced sprout formation in HUVEC spheroids, according to the method published earlier (Korff, Augustin 1999). Also in this model Spermidine and α1-TAT but not scrTAT were able to significantly inhibit VEGF-induced sprouting in HUVECs (III, Fig. 5A). Importantly, the basal sprouting was not affected by Spermidine (III, Fig. 5A). To confirm that these results were caused by the actions of TC45, we silenced it in HUVECs and tested the effect of Spermidine in the presence or absence of VEGF in a similar spheroid assay. Compared to control cells, TC45 silencing (III, Fig. 5D) enhanced VEGF-induced sprouting by $35 \pm 7\%$ (III, Fig. 5C). Furthermore, in TC45 silenced cells spermidine inhibited VEGF-induced sprouting by $43 \pm 5\%$ compared to the $89 \pm 3\%$ inhibition by spermidine in control cells. These results suggest that VEGF-induced sprouting in human primary endothelial cells is inhibited by Spermidine in a TC45-dependent manner.

5.3.5. Activation of TC45 by α1 integrin is controlled by amino-terminal negative residues

We investigated the mechanism behind the integrin α1β1 –mediated TC45 activation. Based on our results, integrin α1 cytoplasmic tail residues 1164-1179 interact with the amino-terminal part of TC45 (amino acids 93-178), activating it by inhibiting the proposed autoregulatory interaction between the carboxy- and amino-terminus of the protein (I). Since α1 cytoplasmic tail is positively charged (RPLKKKMEK), we investigated the presence of negatively charged areas on the surface of the published 3D structure of active TC45 (Iversen et al. 2002). We identified four negatively charged residues glu 8,11,24,28 that form a negatively charged patch in the N-terminus of TC45 (III, Fig. 6A, shown with GKKKG –peptide of α1 tail in yellow), and accordingly created three mutants of TC45. Two mutants contained two point mutations each, glu 8,11 ala (E8,11A) and glu 24,28 ala (E24,28A), and in the third mutant all four glutamates were mutated to alanines (E8,11,24,28A). These mutations had no significant effect on the basal TC45 phosphatase activity (III, Fig. 6D). However, binding of the α1 integrin cytoplasmic tail peptide (α1-cyt) in a surface plasmon resonance assay to E8,11,24,28A-TC45 was $41,4 \pm 7,8$ % lower compared to wt TC45 (III, Fig. 6B). Furthermore, the ability of α1-cyt to activate TC45 was significantly decreased by mutating these 4 negative residues (III, Fig. 6D). To confirm these results, we performed a pull-down assay with biotinylated α1-cytoplasmic tail peptide coupled to streptavidin beads, and recombinant purified and cleaved GST-TCPTP proteins. We found that TC45, both double mutants and truncated TC37 bound to α1-cyt peptide, while no binding was detected with E8,11,24,28A-TC45 (III, Fig. 6C). Thus, the E8,11,24,28A-TC45 mutant was unable to interact with α1-cyt, and also the mutant was not activated by the peptide in an in vitro assay (III, Fig. 6D).

6. DISCUSSION

6.1. The mechanism of TCPTP-mediated downregulation of RTKs by the α1 integrin

We have shown here that α1-cyt binds to N-terminus of TCPTP, and activates it directly and specifically. The C-termini of TCPTP and α1-cyt are both positively charged (Figure 9). We hypothesize that the α1 tail would alleviate the closed, inactive conformation of TCPTP by competing with and replacing the proposed autoregulatory C-terminal segment of the enzyme (Hao et al. 1997) (Figure 9). A similar activation mechanism has been shown for the PTP SHP2, which is maintained in an inactive conformation via an intramolecular association between its N-terminal SH2-domain and the catalytic domain (explained in more detail in 2.2.3.3. Regulation of classical PTP action). The intramolecular interaction is disrupted upon binding of phosphorylated proteins to the two SH2-domains. This leads into a conformational change and activation of SHP2 (Ostman, Hellberg & Bohmer 2006, Mohi, Neel 2007).
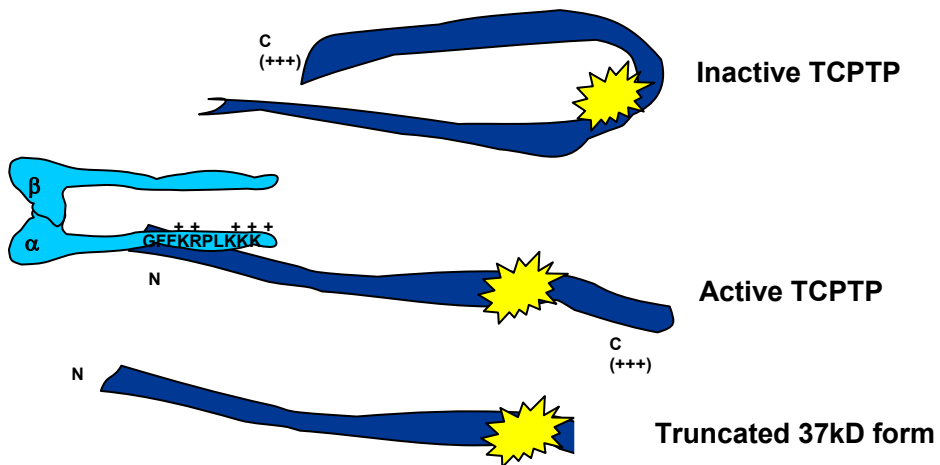

Figure 9. Hypothetical activation model of TCPTP by α1 integrin. In vitro studies have proposed that the catalytic activity of TCPTP is regulated by an intramolecular inhibition involving a carboxy-terminal segment of the 45 kDa form of TCPTP. Association of the inactive TCPTP with the positively charged α1 cytoplasmic tail could alleviate this autoinhibition, competing with the likewise positive C-terminal tail and leading to activation of the phosphatase. The truncated 37 kDa form of the phosphatase lacking the C-terminus is constitutively active because the inhibitory segment is missing.

In the last piece of this work we identified four negatively charged residues glu 8,11,24,28 that form a negatively charged patch at the N-terminus of TCPTP. We mutated all of the four glutamates to alanines, and studied how these mutations affect activation of

TCPTP by α1 integrin cytoplasmic tail (α1-cyt). As a result we found that α1-cyt bound to the mutant in a significantly weaker manner than to the wt TCPTP (III). Also, α1-cyt activated the TC45 containing all the four mutations significantly less (III). Now we have also identified one additional amino acid in the N-terminus of TCPTP that could have an additive effect to the control of TCPTP activation and are investigating that. It will also be interesting to note whether this further mutation renders Spermidine unable to activate TCPTP.

With the present data we are not able to prove that TCPTP activation proceeds as we hypothesize. The detailed structure of the whole enzyme would be essential to be able to verify the mechanism. We have tried to crystallize TCPTP unsuccessfully. For some reason the enzyme is auto-cleaved between the kinase domain and the tail, resulting in the 37 kDa constitutively active form. The activation mechanism can certainly be confirmed only when the crystal structure of the whole enzyme is at hand. Alternatively, the crystal structure of the complex between α1-peptide and 37 kDa TCPTP would be informative.

6.2. TCPTP is activated by integrin α1 cytoplasmic tail to downregulate EGFR

TCPTP activation has been shown to lead to downregulation of five RTKs, namely EGFR (Tiganis et al. 1998, Klingler-Hoffmann et al. 2001), PDGFRβ (Persson et al. 2004a), Met (Sangwan et al. 2008), IR (Galic et al. 2003), and CSF-1R (Simoncic et al. 2006). In this work, we have been able to show that cell binding to collagen and subsequent integrin α1β1 activation leads to TCPTP activation and subsequent EGFR down-regulation (I). As depicted in Figure 10, TCPTP resides in the cytoplasm and the nucleus when inactive. Based on our results, upon cell adherence to collagen or after serum stimulus, TCPTP is translocated to the α1 integrin cytoplasmic tail, which activates the enzyme, leading to dephosphorylation of the RTK. This is remarkable as the first evidence of negative regulation of an RTK by integrins. Previously integrins had been linked only with permissive signalling (Moro et al. 1998, Moro et al. 2002). Moro and co-workers showed that integrins β1 and $α_V$ can activate EGFR in the absence of growth factors (Moro et al. 1998). Here we have shown that integrin α1 is capable of negatively regulating EGFR signalling via activation of TCPTP. This is interesting from the point of view of matrix-dependence of normal, untransformed cells. As the expression of α1 integrin is lost in specific cancers, also the TCPTP-mediated downregulatory system activated by α1 integrin is switched off unless rescued by introducing a TCPTP-activating drug. In addition to providing the first evidence for ECM-derived negative regulation of RTKs, TCPTP activation by the α1 integrin also introduces a new activation mechanism for the PTPs, in addition to the ones presented in the literature review, section 2.2.3. Also, it has not been known before

that in addition to mitogenic stimuli and cellular stress, also α1β1 integrin mediated adhesion to collagen is capable of transporting TCPTP from the nucleus to the cytoplasm (Tiganis et al. 1998, Lam et al. 2001, Sangwan et al. 2008) (I).
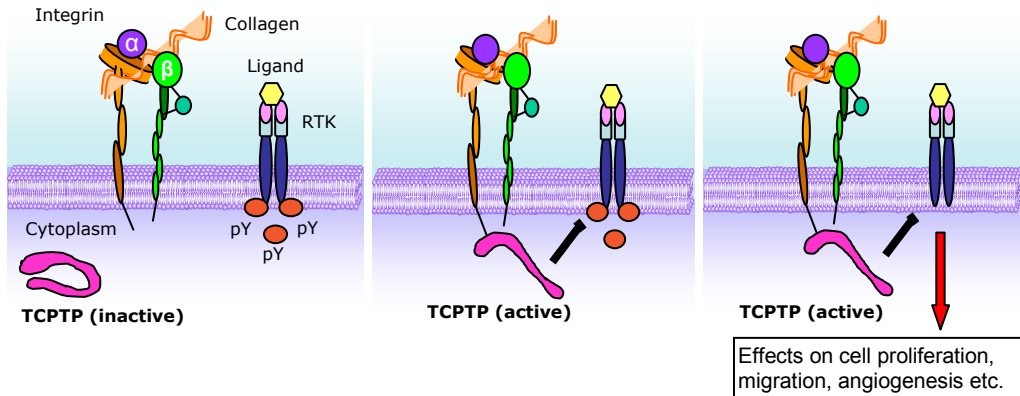

Figure 10. RTK downregulation by integrin α1-activated TCPTP. When inactive, TCPTP locates in the cytoplasm and the nucleus. When cell adheres to collagen or is stimulated by serum, TCPTP translocates to the plasma membrane and comes to physical contact with α1 integrin. There α1 integrin binds to the N-terminus of TCPTP and activates it. TCPTP in turn dephosphorylates the receptor tyrosine kinase, leading to effects in downstream signalling of the receptor.

Chen and coworkers have recently shown that TCPTP downregulation increases basal levels of EGFR in mesangial cells found in the glomerulus of the kidney (Chen et al. 2007), indicating that TCPTP regulates EGFR activation also in mesangial cells. They also found that in α1-knockout mesangial cells EGFR phosphorylation was upregulated, supporting our findings (I). Chen and others concluded that TCPTP is a key player in control of α1 integrin –dependent EGFR-activation in mesangial cells.

Chen and coworkers also found that TCPTP regulates production of ROS in the kidney (Chen et al. 2007). According to their results, in the absence of integrin α1β1, TCPTP is not recruited to downregulate EGFR, leading to increased EGFR phosphorylation and subsequent phosphorylation of Vav2, a guanine nucleotide exchange factor (GEF) for the Rho/Rac family of small G proteins. Vav2 in turn activates Rac1, leading to its translocation to the cell membrane. This results in increased production of ROS, probably by activation of NADPH oxidase, and increased collagen IV production. Positive feedback loop is closed by ROS possibly inducing increased EGFR phosphorylation and increased collagen IV production (Chen et al. 2007).

6.3. TCPTP as a regulator of angiogenesis and lymphangiogenesis

6.3.1. A new RTK recognised as a TCPTP substrate

We have identified a previously unrecognised connection between VEGFR2 and TCPTP (II). In our studies, we were able to show that TCPTP substrate-trapping mutant interacts with VEGFR2 and dephosphorylates it site-specifically on tyrosines 1054/1059 and 1214. The first two are needed for maximal activation of the kinase activity of the receptor (Claesson-Welsh 2003) and Tyr1214 triggers the p38 cascade through unknown intermediates (Olsson et al. 2006). In addition we showed that TCPTP activation decreases internalisation of VEGFR2, chemotaxis, and VEGF-driven sprouting angiogenesis in HUVECs, whereas silencing of TCPTP enhances the proliferation of HUVECs. Importantly, TCPTP was co-immunoprecipitated with VEGFR1 as well, but only in vitro, underscoring the importance of testing protein-protein interactions also in vivo.

Of the family of PTPs, previously only HCPTPA, a low molecular weight, cytoplasmic PTP, has been shown to regulate VEGFR2 signalling (Huang et al. 1999). Huang and co-workers found the interaction using Yeast-two-hybrid method to screen human fetal heart library with VEGFR2 kinase domain as the bait. HCPTPA bound specifically to the active, autophosphorylated VEGFR2 but not to a mutated, kinase-inactive VEGFR2. Recombinant HCPTPA was shown to be able to dephosphorylate VEGFR2 in vitro and in vivo. Site-specificity of the dephosphorylation was not examined, perhaps because of the lack of suitable site-specific VEGFR2 phospho-antibodies at the time of the experiments. Huang and others also showed that overexpression of HCPTPA greatly reduced VEGF-mediated survival/proliferation and migration in primary endothelial cells, HUVECs, as well as blocked the formation of vascular sprouts in a VEGF-dependent angiogenesis model.

The fact that in vitro also VEGFR1 was bound by TCPTP in an immunoprecipitation assay demonstrates both the specificity of TCPTP and underscores the importance of using test systems that are as close to physiological conditions as possible. In addition to VEGFR2 and VEGFR1, also VEGFR3, which regulates lymphangiogenesis, belongs to the VEGF-receptor family. The structures of TCPTP substrate VEGFR2 and VEGFR3 are very similar (Figure 11 bottom), with about 80 % identity to VEGFR1 in the tyrosine kinase domain (Shibuya, Claesson-Welsh 2006, Roskoski 2008). A distinctive feature in VEGFR3 is the substitution of one of the seven IgGs present in VEGFRs 1 and 2 to a disulfide bridge that holds the proteolytically cleaved N-terminal part of the extracellular domain connected with the rest of the molecule (Pajusola et al. 1994).
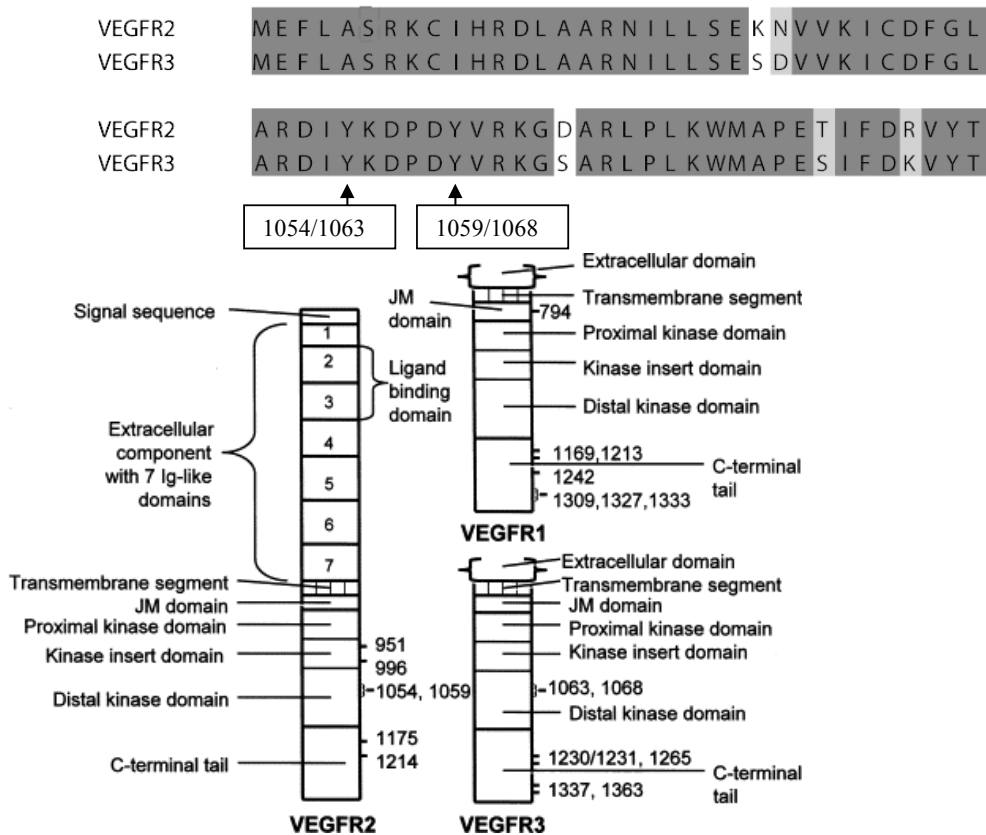
VEGFR2 M E F L A S R K C I H R D L A A R N I L L S E K N V V K I C D F G L
VEGFR3 M E F L A S R K C I H R D L A A R N I L L S E S D V V K I C D F G L

VEGFR2 A R D I Y K D P D Y V R K G D A R L P L K W M A P E T I F D R V Y T
VEGFR3 A R D I Y K D P D Y V R K G S A R L P L K W M A P E S I F D K V Y T

1054/1063   1059/1068

Figure 11. Organization of the VEGFRs. (Top) Alignment of amino acid sequences between VEGFR2 (amino acids 1016-1083) and VEGFR3 (amino acids 1025-1092). The tyrosines 1054 & 1059 (VEGFR2) and 1063 & 1068 (VEGFR3) have been marked with arrows. (Bottom) Numbers on the right of each receptor correspond to human tyrosine residue phosphorylation sites. The relative lengths of the receptor components are to scale. Bottom picture reproduced from (Roskoski 2008) with permission of the author.

The VEGFR2 distal kinase domain phosphorylation site Tyr1054/1059, which we found to be downregulated by TCPTP, corresponds to the Tyr1063/1068 of VEGFR3, and in fact they are very similar in sequence (Figure 11 top). On the contrary, very little similarity in sequence is found between another site on VEGFR2 affected by TCPTP, Tyr1214 in the C-terminal tail, and Tyr1230/1231 on VEGFR3 (not shown) (Roskoski 2008). The very similar sequence between VEGFR2 Tyr1054/1059 and VEGFR3 Tyr1063/1068 implies that also VEGFR3 could be a TCPTP substrate.

6.3.2. General model for phosphatase-regulated angiogenesis

Our findings together with recent reports on the role of two other phosphatases, DEP-1 and SHP2, in controlling VEGFR2 (Lampugnani et al. 2006, Mitola et al. 2006) allows us to propose a general model of sequential, phosphatase-regulated steps in angiogenesis (Figure 12). (i) In intact endothelium, cells are contact-inhibited and unable to respond

to VEGF. This is regulated by VE-cadherin-β-catenin complex that targets VEGFR2 to cell-cell contacts and results in its dephosphorylation by DEP-1 and possibly by other junctional phosphatases. In these confluent cells, ligation of α1β1 to basement membrane collagen IV and subsequent TCPTP activation would not have any additional effect since VEGFR2 is already functionally inactivated through rPTP DEP-1.
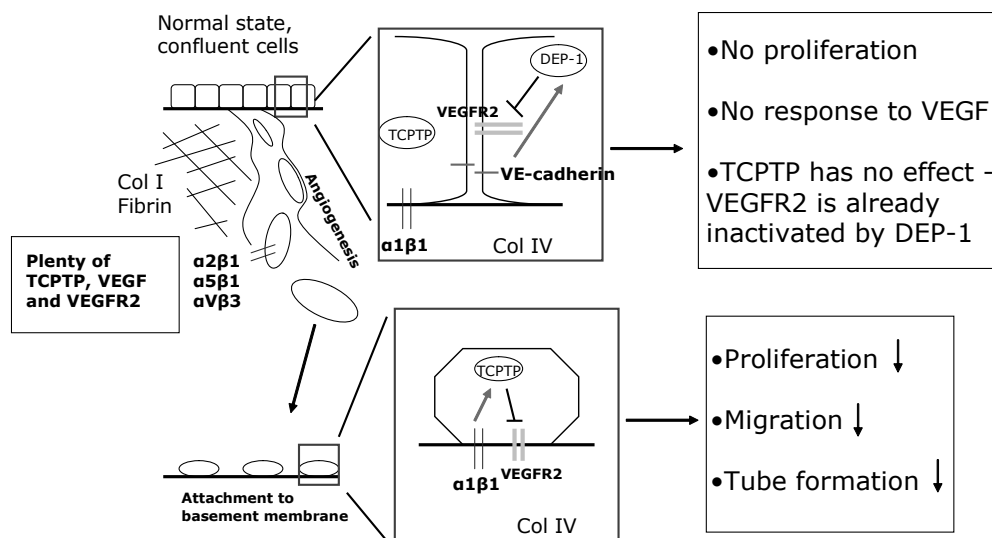

Figure 12. Different phosphatases inhibit VEGF signalling at different steps of angiogenesis.

(ii) Upon induction of angiogenesis, the sprouting endothelial cells become surrounded by stroma ECM and are ligated most likely via integrins αvβ3, α5β1, and α2β1 (which bind with high affinity to vitronectin, fibronectin and type I collagen, respectively). The cells are sensitive to VEGF, and VEGFR2 is fully phosphorylated and actively signalling to drive vessel sprouting. (iii) Upon formation of a new basement membrane, α1β1 integrin (binding to collagens IV and I) will activate TCPTP, and possibly SHP2, which begin to attenuate proliferation and migration of the endothelial cells until they reach confluency and become contact-inhibited (i). Based on this model, different phosphatases would inhibit VEGF signalling at different steps of angiogenesis.

6.4. TCPTP as a drug target

TCPTP is expressed ubiquitously in all tissues, and, unlike e.g. PTEN, it is not lost in cancers, which makes TCPTP an interesting target for drug development. This is unusual, as the expression of several other tumor suppressors is lost in transition from benign to malignant. Also, it is questionable whether TCPTP can be classified as a bona fide tumor suppressor, since its expression is not lost in cancer. The α1 integrin in turn is expressed in endothelial cells (Abair et al. 2008). Interestingly, the expression is lost in specific cancers still expressing TCPTP. Nevertheless, in these specific tumors having

lost their α1 integrin expression, reintroduction of the integrin could possibly have tumor suppressive effects via TCPTP.

Challenges to the development of a selective and effective TCPTP-activator are brought e.g. by the fact that the mechanism of α1-mediated activation of TCPTP is not yet fully resolved. We have here identified the putative binding site for α1-cyt in the negatively charged patch of TCPTP N-terminus. However, this information is still not sufficient to determine the exact way that α1-cyt binds to TCPTP and activates it. As already mentioned above, we are now working on a fifth point mutation that could potentially help in modelling the binding mechanism. At present it is unclear whether the small molecule activators bind to this same site or activate TCPTP via a different mechanism. Thus, more mutagenesis data are needed to precisely determine the binding site of α1 on TCPTP that would allow design of novel TCPTP activators binding specifically to this site. Also, the recent findings by Barr and coworkers (Barr et al. 2009) on the presence of a secondary substrate-binding pocket in TCPTP could be of use in designing novel TCPTP activators, at the least in ensuring that both the primary and secondary pockets are well available for TCPTP substrates after activation of the enzyme. Other challenges in the drug development process will very likely be caused by affinity, specificity, solubility, and problems related to drug administration, distribution, metabolism and excretion (ADME). Also the high homology between TCPTP and PTP1B has to be taken into account when designing a TCPTP-specific activator.

We have already performed several in vivo experiments to evaluate the ability of the α1-peptide to inhibit tumor growth in mice. We injected α1-null HT1080 –cells into nude mice and treated the developing tumors with either α1- or scrTAT –peptides regularly. The initial results indicated that the α1-TAT –treated tumors were necrotic and lacked vessels, but for some reason the significant result was repeated only as a trend in subsequent experiments using larger group sizes of mice. There are two obvious problems with this study design that could explain the variability of the results. First, the stability of the TAT-peptides in vivo is not known and a short half-life due to proteolysis could limit their efficacy. In addition, another cell line with a more pronounced dependence on EGFR signalling might have been more suitable to TCPTP activation and would thus have been a better choice. Nevertheless, the ability of TCPTP to inhibit VEGF signalling in vitro could be further tested with e.g. the Matrigel plug –method in mice, applying a TCPTP-activator into the plug together with VEGF and assessing the effect on angiogenesis.

Although Spermidine was identified in this study as a TCPTP-activator, we do not intend to use it as such as a drug candidate, but instead use it and the results achieved by using it to develop a novel, small-molecule activator of TCPTP. This putative TCPTP activator could, for example, be used in cancer patients that are or have become resistant to EGFR inhibitors, and in combination treatment with other anti-cancer therapies.

7.   SUMMARY / CONCLUSIONS

In this thesis study the actions of TCPTP on several RTKs have been elucidated. Via activation by α1 integrin cytoplasmic tail, TCPTP has been shown to downregulate EGFR signalling after cell adhesion to collagen. This activation of TCPTP inhibited EGF-induced cell proliferation and anchorage-independent growth of malignant cells. These data provide the first evidence of negative regulation of an RTK by integrins, which have previously been linked only with permissive signalling (Moro et al. 1998, Moro et al. 2002).

The results identified TCPTP as a negative regulator of VEGF-signalling via VEGFR2 for the first time. TCPTP was shown to be site-specific in its responses towards VEGFR2. TCPTP also inhibited VEGFR2 kinase activity and prevented receptor internalization from the cell surface. Activated TCPTP was shown to inhibit VEGF-induced endothelial cell proliferation, angiogenic sprouting, chemokinesis and chemotaxis.

In addition, we have identified a novel and specific TCPTP activator, spermidine, by a High-throughput screen against 64,280 compounds. Spermidine was shown to be able to downregulate both EGFR and PDGFRβ phosphorylation in cells, as well as to attenuate VEGF-induced sprouting of endothelial cells in a TCPTP-dependent manner. Last, we investigated the mechanism by which α1β1 integrin activates TCPTP. We created a TCPTP mutant in which the putative binding site of α1-cyt was altered. The mutations did not alter the basal phosphatase activity of TCPTP, but instead decreased the ability of α1-cyt to bind and activate TCPTP.

In conclusion, in this study we have been able to show that α1 integrin activates TCPTP to downregulate signalling of RTKs EGFR and VEGFR2 in a site-specific manner. α1-cyt has here been demonstrated to control several crucial functions such as proliferation and anchorage-independent growth of malignant cells, as well as to regulate growth, migration and differentiation of human endothelial cells. Also, we have been able to provide important information about TCPTP activation by α1 integrin cytoplasmic tail, which is in fact the first detailed characterization of the activation of TCPTP to date.

The results of this study improve the understanding of the molecular mechanisms behind TCPTP activation, and encourage to further develop TCPTP activators that could one day be in clinical use against diseases such as cancer.

8. ACKNOWLEDGEMENTS

Kulta is recognised for the many occasions that he offered his helping hand in computer-related and other issues. Also I want to thank Sirkku Grönroos, Eva Hirvensalo, and Aila Jasmavaara at the BTK office, as well as Outi Irjala and Riitta Paju at the medical faculty office.

The people working in other research groups at VTT Turku are thanked for providing a cheerful and motivating working environment. The joyful discussions as well as the scientific change of ideas at the corridors and during coffee breaks have been helpful and relaxing.

I also want to acknowledge the people who provided various constructs, cells, antibodies, services, and scientific support for my work. These people include Professor Jyrki Heino, Professor Michel Tremblay, Professor Lena Claesson-Welsh, Docent Sanna Käkönen, Dr. Tony Tiganis, Professor Peter J. Parker, Dr. Ambra Pozzi, Dr. Jo Durgan, and Camilla Nylund.

My friends outside of work are thanked for great company, support and for being there.

I want to thank my family for support throughout my life. My sincerest thanks go to my Mom, whose help especially in the form of taking care of Aapo while I was writing this thesis has been irreplaceable. Last, I want to thank my boys Aki and Aapo for existence, love and support. Aapo, you are the Sunshine of my life.

Turku, March 2009

Elina Mattila

9.   REFERENCES

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