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Proteolytic Processing of the Receptor-like Protein Tyrosine Phosphatase κ and Deregulation in Human Cancer

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„Ist das Erfinden Sache der Genialen, so ist die treffende Wahl Sache der Verständigen. Eine treffende Wahl gelingt vielen, eine gute Erfindung wenigen, und zwar nur den ersten, dem Wert und der Zeit nach“

HANDORAKEL UND KUNST DER WELTKLUGHEIT
Balthasar Gracian (1601-1658)

Contents

I. Introduction

1. Protein tyrosine phosphorylation – a central mechanism in signal transduction

The millions of cells that constitute an organism always communicate with each other, close or at a distance. By doing so, they build up an extremely complex signaling network in order to precisely coordinate all their functions. Such signaling systems are balanced to give the exact response in every given situation. An interrupted or perturbed system, with signaling components missing or unable to respond, or a system out of tune, will unconditionally lead to a failed response.

An external signal is often received by receptors at the surface of the cell. These receptors specifically recognise the signal and will activate the intracellular signaling network corresponding to the particular signaling molecule. Tyrosine phosphorylation of a protein is a central mechanism in these signaling networks. Protein tyrosine phosphorylation may lead to a change in catalytic activity, if this protein is an enzyme, or may provide a binding site for other proteins, thus causing specific protein-protein interactions. Tyrosine phosphorylation is reversible and regulated by the co-ordinated and competing actions of two enzyme families: protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) (Hunter, 1992; Hunter, 1995; Fischer, 1999). Both families contain proteins that are structurally diverse and include both receptor-like and cytoplasmic enzymes. The striking variation in structure presumably reflects the wide range of different interactions in which these proteins participate.

The involvement of tyrosine phosphorylation in cellular signaling was first identified in tumor cells transformed by virus. The transformed cells, showing morphological changes and unregulated growth, exhibit a significant increase in protein tyrosine phosphorylation (Eckhart et al., 1979; Hunter and Sefton, 1980; Witte and Cornicelli, 1980). The role of tyrosine phosphorylation in cell growth was further proven by the finding that growth factors induce tyrosine kinase activity after binding to their receptors (Ek et al., 1982; Kasuga et al., 1982; Ushiro and Cohen, 1980; Cohen, 2002). It has also later been shown that signaling through the receptor-like protein tyrosine kinases (RTKs) may in some cases induce differentiation rather than growth (Marshall, 1995).

The eventual outcome of a ligand binding to its receptor-like tyrosine kinase is complex, not only dependent on the identity of the ligand, but also on the state of the cell and sum of other signals received by the cell (Marshall, 1995). Furthermore, tyrosine phosphorylation has been shown to be a key regulator of many different cellular processes such as growth control, differentiation, cell shape and migration, gene transcription and synaptic transmission (Hunter, 1998a, 1998b).

According to the central role of tyrosine phosphorylation in cellular processes, it has been shown that functional perturbation of tyrosine kinases and tyrosine phosphatases underlies many diseases. Overexpressed or mutationally activated forms of at least ten tyrosine kinases have been implicated in human cancers (Blume-Jensen and Hunter, 2001). Mutations of RTKs may result in developmental dysfunction (Vikkula et al., 1996; Webster and Donoghue, 1997; Robertson et al., 1997, 2000), diabetes (Kahn et al., 1996; Taylor et al., 1992) and immunodeficiencies (Tsukada et al., 1994). Mutations in PTPs also play a role in disease (Shultz et al., 1997; Tsui et al., 1993; Tartaglia et al., 2001; Ruivenkamp et al., 2002), but the signaling functions of these enzymes and their mode of regulation are much less characterized (Li and Dixon, 2000).

2. Protein tyrosine phosphatases – a structurally and functionally diverse family of enzymes

Protein tyrosine phosphatases are characterized by the conserved, approximately 240 amino acid long PTPase domain, which is defined by presence of the PTP active site signature sequence (I/V)HCXXGXXR(S/T) (Fischer et al., 1991). The PTPs constitute a structurally diverse family of enzymes consisting of three subclasses: classical tyrosine-specific PTPs, low molecular weight PTPs (LMW-PTPs) and dual-specific PTPs (DSP-PTPs) (van Huijsduijnen et al., 1998; Andersen et al., 2001b). The LMW-PTP family comprises 18 kD enzymes with specificity primarily towards phosphotyrosine. Dual-specific PTPs (DSPs) are capable of dephosphorylating serine- and threonine residues in addition to tyrosine residues. Examples of the DSP family are the MAP kinase phosphatases (MKPs), the CDK phosphatases Cdc25 and KAP (kinase-associated phosphatase), VH1 of vaccinia virus and the phosphatidylinositol 3,4,5-triphosphate phosphatase PTEN (*p*hosphatase and *ten*sin homolog deleted on chromosome *ten*).

Based on vertebrate sequence data of 113 PTP catalytic domains, 17 principal PTP subtypes were identified (Fig. 1; Andersen et al., 2001b). To date, 37 distinct human members of the classical tyrosine-specific PTP family have been described. The classical tyrosine-specific PTPs encompass both transmembrane receptor-like and cytosolic enzymes.

The majority of the receptor-like PTPs (RPTPs) contain two catalytic domains: a membrane proximal domain (D1), mainly responsible for catalysis, and a membrane distal domain (D2), containing little or no phosphatase activity (Wang and Pallen, 1991; Wu et al., 1997). In fact, some D2s lack residues that are essential for catalysis; for example, the highly conserved cysteine residue in D2 of PTPβ/ζ and PTPγ is replaced by an aspartic acid residue (Barnea et al., 1993; Krueger et al., 1990). Nevertheless, D2s are highly conserved and replacement of only two residues in PTPα D2 with those that are present in PTPα D1 potentiates PTP activity to levels that are comparable to D1 (Lim et al., 1998; Buist et al., 1999). Given the low PTP activity displayed by D2 it is unlikely that this domain dephosphorylates substrate proteins in the cellular context (den Hertog, 1999). Instead, it has been suggested that RPTP-D2s bind and are proposed to regulate RPTP-D1 activity involving both, intramolecular and intermolecular interactions (see below, Blanchetot et al., 2002a, 2002b; Wallace et al., 1998; Feiken et al., 2000, Aricescu et al., 2001; Felberg, 1998). Furthermore, RPTP-D2s have been implicated in binding to potential effector proteins like Grb2 and liprins (den Hertog et al., 1994; Serra-Pages et al., 1995).

In addition to the phosphatase domains, RPTPs are composed of a single transmembrane segment and an extracellular part. The extracellular portion exhibits a broad structural variation, ranging from very short to very extensive (Fig. 1). For instance, RPTP subtype R4, consisting of PTPα and PTPε, is characterized by a short and heavily glycosylated extracellular part, e.g. 27 amino acid residues in case of PTPε. Importantly, PTPα, by dephosphorylating and activating src family of PTKs, can modulate signal transmission in a positive manner (Zheng et al., 1992; den Hertog et al., 1993; Ponniah et al., 1999; Su et al., 1999), pointing out the fact that PTPs not only serve housekeeping functions as negative regulators of cell signaling. RPTP subtype R1/6 includes CD45, which is composed of a heavily glycosylated fibronectin type III-like domain. CD45, the first RPTP to be identified (Charbonneau et al., 1988), is specifically expressed in lymphocytes and plays an essential role in T-cell receptor-initiated signal transduction and lymphocyte development (Hermiston et al., 2003). As reported for PTPα, the most prominent substrates of CD45 are the src family

PTKs, which become activated upon dephosphorylation of the carboxy-terminal inhibitory tyrosine. The PTP LAR-family (R2B) contain fibronectin type III domains and immunoglobulin-like domains. Both domain types are also present in certain members of the

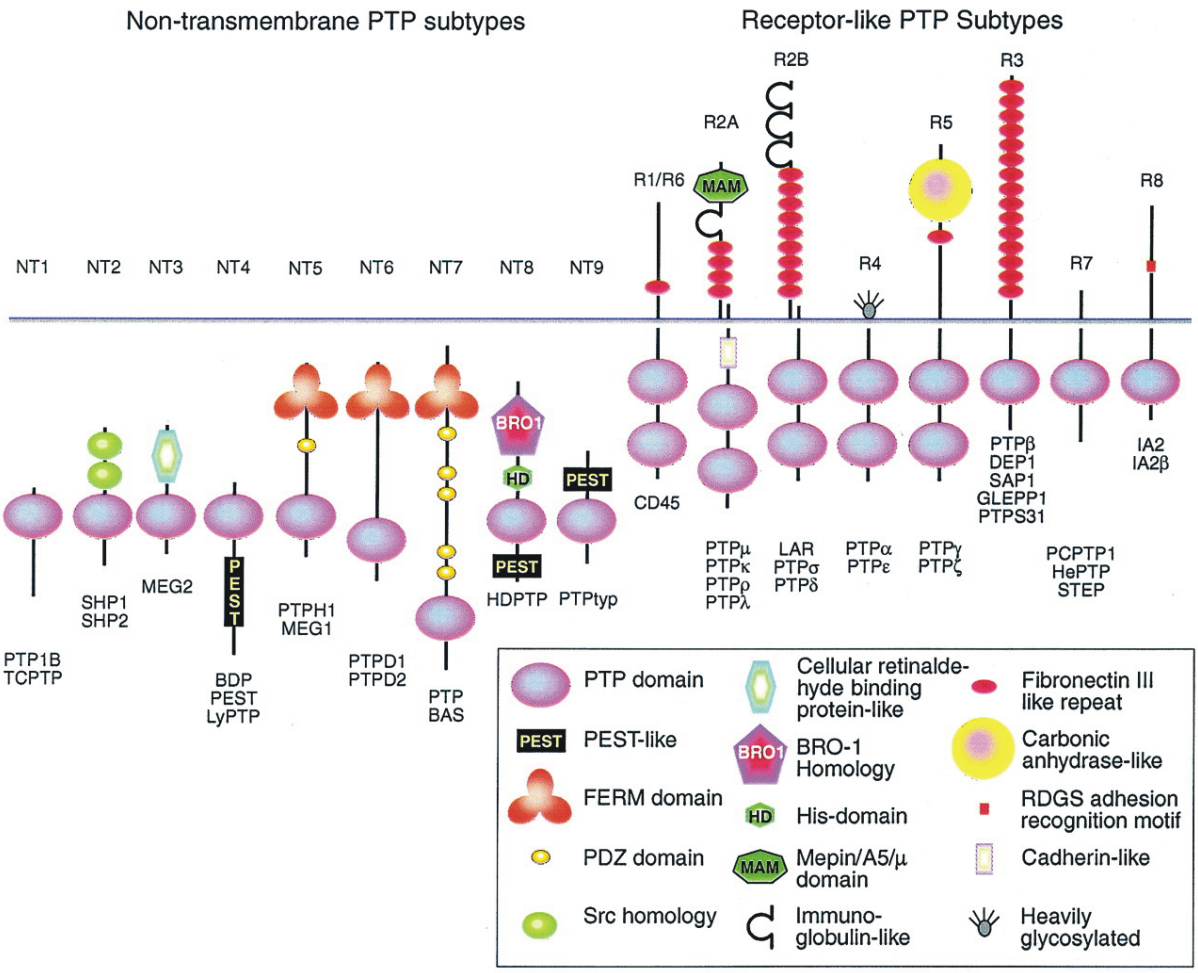

Figure 1: Schematic representation of PTP family members. *Determination of sequence similarity among PTP catalytic domains (Andersen et al., 2001b) was used to classify the PTP family of enzymes into 9 nontransmembrane subtypes (NT) and eight RPTP subtypes (R). Only human PTPs are listed, and a representative member of each subtype is shown (Andersen et al., 2001b).*

immunoglobulin superfamily of cell adhesion molecules, e.g. N-CAM and Ng-CAM. In addition to fibronectin type III domains and immunoglobulin-like domains, the MAM-family of PTPs, including PTPκ, PTPμ, PCP-2 and PTPρ contain a MAM domain at their N-terminus and constitute type R2A-PTPs. Some structural features of the RPTP extracellular parts, such as immunoglobulin-like domains and fibronectin type III domains, are similar to cell adhesion molecules, suggesting that RPTPs might be involved in cell-cell and cell-extracellular matrix interactions (Brady-Kalney and Tonks, 1995). Indeed, members of the MAM-family of PTPs are localized at sites of cell-cell contact, while PTP-LAR has been

observed preferentially at points of cell-extracellular matrix attachment. A typical feature of the MAM domain-containing RPTPs is their 70 amino acid residue long, extended juxtamembrane region with sequence similarity to the juxtamembrane part of cadherins. This region is thought to mediate binding to ß-catenin (see below). Type R3 PTPs are DEP-1 and PTPβ, which are composed of multiple fibronectin type III domains. In analogy to the MAM and LAR family of PTPs, the protein expression of DEP-1 strictly depends on cell density, e.g. RPTP expression increases with increasing cell density (Östman et al., 1994; Gebbink et al., 1995; Fuchs et al., 1996; Symons et al., 2002), again implicating RPTPs in the regulation of signaling processes that are initiated by cell contact. PTPζ/β is the prototypic member of the R5-PTP subtype and contains an amino-terminal carbonic anhydrase domain. The diversity of the extracellular domains of RPTPs suggests that they serve as specific ligand binding sites and several ligands for PTPζ/β have been identified (see below).

Cytosolic PTPs contain one single catalytic domain and additional flanking regions with putative roles in regulation of catalytic activity, protein-protein-interactions and subcellular targeting (Tonks and Neel, 2001). SHP-1 and SHP-2 contain SH2 domains, which are involved in both activity regulation and substrate binding (Feng and Pawson, 1994; Van Vactor et al., 1998). Importantly, the biological functions of SHPs are totally different, even though they are highly related (60% sequence identity, type NT2). For example, SHP-1 acts as negative regulator of several hematopoetic signaling pathways, including cytokine, growth factor, adhesion and antigene receptor-initiated signaling (Klingmüller et al., 1995; You et al., 1997; Neel, 1997). In contrast, SHP-2 most often functions as positive regulator of signal transmission, in particular of growth factor signaling initiated by EGF, FGF, IGF-1, PDGF and intergrin signaling (Oh et al., 1999; Qu et al., 1999; Saxton et al., 1997; Shi et al., 1998; Yu et al., 1998). LyPTP and PTP-PEST contain PEST domains, suggested to have a role in substrate targeting. PDZ domains have been reported to bind to specific hydrophobic sequences in the C-termini of substrate proteins and are found for instance in PTP-BAS (Saras and Heldin, 1996). PTP1B and TC-PTP share high sequence similarity (type NT1) and contain a carboxy-terminal endoplasmic reticulum-targeting domain. Subcellular targeting of PTPs by flanking domains will be discussed below. Both PTPs are known to antagonize RTK-initiated signaling. The most prominent substrate for PTP1B is the insulin receptor and mice deficient in PTP1B are insulin-sensitiv (Elchebly et al., 1999, 2000).

Three-dimensional structures, together with kinetic analysis, have provided a clear picture of the catalytic mechanism of PTPs (Barford et al., 1998). The catalytic domains of five cytosolic PTPs (PTP1B, Yop51, SHP-1, SHP-2 and TC-PTP) and four RPTPs (PTPα, PTPμ, LAR and PTP-SL) have been crystallized to date (Barford et al., 1994; Bilwes et al, 1996; Hof et al., 1998; Hoffmann et al., 1997; Iversen et al., 2002; Nam et al., 1999; Stuckey et al., 1994; Szedlacsek et al., 2001; Yang et al, 2000).

Central in the catalytic mechanism used by PTPs to remove phosphate from phosphotyrosine residues in proteins is the cysteine in the signature motif, residing at the bottom of the active site cleft (Guan and Dixon, 1991; Barford et al., 1998a). The cleft is surrounded by four loops, which contain residues essential for catalysis and substrate recognition. The depth of the cleft confers specificity towards phosphotyrosine since hydrolysis of the shorter phosphoserine and phosphothreonine residues is prevented (Jia et al., 1995). Binding of substrates induces a conformational change of one of the loops, thus closing the cleft and trapping the phosphotyrosine close to the active site.

The reaction is performed in two steps and involves the formation of a cysteinyl-phosphate intermediate followed by hydrolysis of the intermediate (Pannifer et al., 1998). The cysteinyl-phosphate intermediate is formed through a nucleophilic attack on the phosphate by the unprotonated catalytic cysteine residue (Fig. 2A). An invariant protonated aspartic acid in the flexible loop enhances cleavage of the P-O bond by acting as a general acid catalyst. The intermediate is then hydrolysed by a water molecule, which is activated by the aspartic acid now acting as general base (Fig. 2B). Substitution of the catalytic cysteine residue for a serine or alanine abolishes catalytic activity and the ability to form an intermediate (Guan and Dixon, 1991). Substitution of the catalytical aspartic acid for an alanine allows the enzyme to form stable complexes with the subtrate protein *in vivo* (Flint et al., 1997). These so called "substrate trapping" mutants have been used to identify physiological substrates of PTPs.

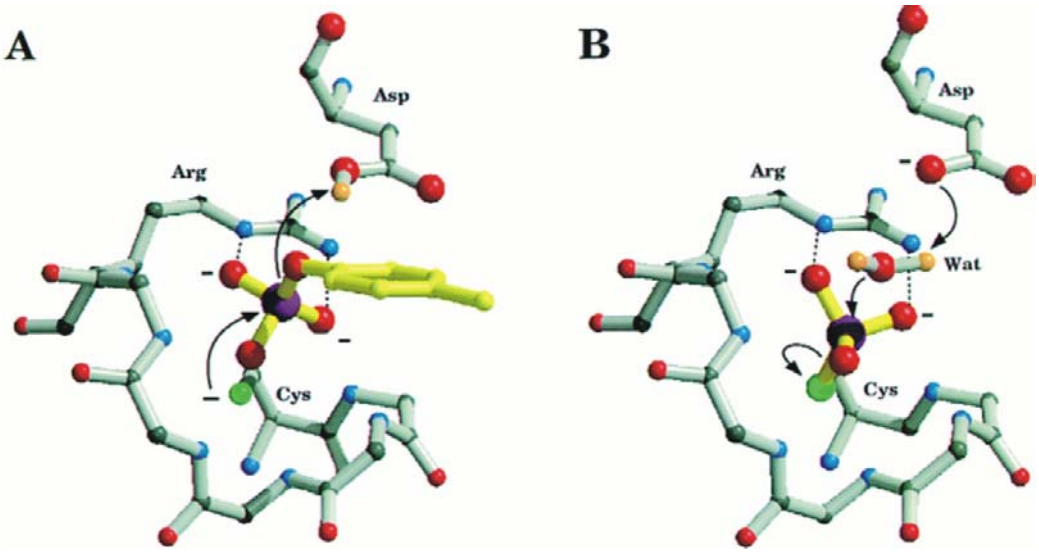

Figure 2: The two-step dephosphorylation mechanism of protein tyrosine phosphatases. *Models of the enzyme-substrate complex of PTP derived from two X-ray crystallographic models: Cys-ser mutant of PTP1B complexed with phosphotyrosine (Jia et al., 1995), and Yersinia PTP complexed with vanadate (Denu et al., 1996a). A. Formation of the cysteinyl-phosphate intermediate. The backbone atoms of the active site loop from cysteine to arginine are shown as a ball-and-stick model. The phosphotyrosine substrate is shown in yellow. The dianion of the phosphoryl group is coordinated by the nitrogens of the arginine side chain and by the amide groups of the active site motif. The thiolate anion from the active site cysteine attacks the phosphate of the substrate and forms a cysteinyl-phosphate intermediate. Expulsion of phospho-tyrosine is aided by the protonated general acid (Asp) of the enzyme. B. Hydrolysis of the cysteinyl-phosphate intermediate. A water molecule (Wat) activated by the general base (Asp) attacks the intermediate (Denu et al., 1996b).*

3. Regulation of protein tyrosine phosphatases

3.1. Subcellular targeting

The regulation of tyrosine phosphatase activity may be thought of as multilevel mechanism. Certainly it is a question of the amount of phosphatase expressed. It is also dependend on the localization of the PTP and on its stability to physically interact with its substrate. Finally, there is a possibility of regulating the specific enzymatic activity.

Several PTPs contain structural features that localize them to certain positions in the cell. PTP1B contains a hydrophobic carboxy-terminal tail, which confers localization to the endoplasmatic rediculum (Frangioni et al., 1992), hence contributing to limited substrate recognition. Consisitent with its targeting domain, PTP1B-catalyzed dephosphorylation of EGFR and PDGFR occurs at specific locations on the surface of the endoplasmatic reticulum (Haj et al., 2002). In addition, PTP1B contains an aminoterminal region conferring specific binding to the insulin receptor (Dadke et al., 2000). In PTP-PEST, sequences carboxy-terminal of the catalytic domain bind to paxillin and the paxillin homologue Hic-5, both

localized at focal contacts (Nishiya et al., 1999; Shen et al., 2000). PTPH1, PTPBAS, PTPMEG and PTPD1 contain FERM domains (Gu and Majerus, 1996; Maekawa et al., 1994; Moller et al., 1994; Zhang et al., 1995). These domains mediate targeting of cytoskeleton associating proteins to cytoskeletal-membrane interfaces (Arpin et al., 1994; Bretscher et al., 1999). The presence of FERM domains in PTPs indicates targeting to cytoskeleton- and membrane associated proteins, which might be putative substrates for dephosphorylation. Alternative splicing generates two forms of TC-PTP (TC45 and TC48) that differ in their carboxy-terminal motifs. As a consequence, TC45 is targeted to the nucleus while TC48 is targeted to the endoplasmatic reticulum, respectively (Lorenzen et al., 1995). SHP1 and SHP2 are two well-characterized PTPs, which contain SH2-domains mediating recruitment to the appropriate tyrosine phosphorylated protein (Feng, 1999; Zhang et al., 2000). The SH2-domains of SHP-1 and SHP-2 have an additional regulatory function. When the PTP is not bound to a substrate, the amino-terminal SH2-domain occupies and inactivates the catalytic site. Upon binding of the carboxy-terminal SH2-domain to a tyrosine-phosphorylated substrate, a conformational change disrupts the interaction and activates the PTP (Barford et al., 1998b; Hof et al., 1998; Yang et al., 2000).

Receptor-like PTPs are by definition embedded in the cell membrane and domains of the RPTP extracellular fragments can localize these enzymes to sites of cell-cell or cell-matrix contacts. For instance, the PTPκ/μ subgroup has been found to interact in a homophilic manner at sites of cell-cell interactions (Brady-Kalnay et al., 1993; Gebbink et al., 1993; Sap et al., 1994). This interaction may cause local upregulation of PTP-activity due to the amount of phosphatase molecules trapped at cell-cell contacts (Gebbink et al., 1995).

3.2. Proteolytic processing

Several studies indicate that proteolytic cleavage is a mechanism inducing cellular redistribution of PTPs. In some cases this process is associated with changes in phosphatase activity. Calpain-catalyzed cleavage of PTP1B results in relocalization from membranes to the cytosol concomitant with a two-fold increase in catalytic activity (Frangioni et al., 1993; Rock et al., 1997). “Proteolytic activation” of PTP-MEG has been shown to be mediated by calpain (Gu and Majerus, 1996). PTP-STEP is proteolytically cleaved by calpain, leading to the release of smaller PTP-isoforms (Nguyen et al., 1999; Gurd et al., 1999). Calpains are cytosolic cysteine proteases that are activated by a rise in intracellular calcium.

Regulated proteolysis has also been demonstrated for RPTP subtypes R2A, R2B and R4. Proteolytic processing of R2A and R2B PTPs by furin- and subtilisin-like proteases in the secretory pathway yields two-subunit enzymes (Fig. 1., Streuli et al., 1992; Jiang et al., 1993). Induced proteolysis of LAR and PTPδ at an extracellular juxtamembrane site was shown to result in shedding of the extracellular fragment, subsequent internalization, and redistribution of the transmembrane part away from sites of cell-cell contact (Aicher et al., 1997). RPTP shedding represents a potential regulatory event not yet fully understood. The localization of proteolytic extracellular fragments seems to be restricted to regions of tight cell-cell interactions (Brady-Kalnay and Tonks, 1994; Jiang et al., 1993; Streuli et al., 1992; Yan et al., 1993). Furthermore, shedding of the processed extracellular fragments containing homotypic binding motifs has been suggested to antagonize interactions of RPTPs expressed on the surface of adjacent cells (Brady-Kalnay and Tonks, 1995).

Naturally occurring cytoplasmic forms of PTPα are generated by calpain-mediated cleavage at an intracellular juxtamembrane site (Gil-Henn et al., 2001). The translocation impaired the ability of PTPα to dephosphorylate substrate proteins associated with the membrane, such as src. Membrane localization of PTPε has also been shown to be crucial for attenuating insulin signaling (Andersen et al., 2001c). Three different non-receptor isoforms of PTPε are generated by initiation of translation at an internal initiation codon of PTPε mRNA molecules, by calpain-mediated proteolytic processing of larger PTPε proteins or by transcription from an alternative promotor (Elson and Leder, 1995; Gil-Henn et al., 2000, 2001). In the first two cases, the generated PTPε isoforms are exclusively cytoplasmic, whereas the alternatively transcribed isoform (at-PTPε), which harbors an additional stretch of amino acids at the N-terminus, is predominantly cytoplasmic but can also be detected in the cell membrane and within the cell nucleus. The N-terminal amino acids of at-PTPε are critical for its nuclear localization and increased oxidative stress enhances its accumulation in cell nuclei (Kraut et al., 2002).

3.3. Ligand binding

To date, only one “classical” soluble ligand for RPTPs has been identified. The finding that pleiotrophin inhibits PTPβ/ζ catalytic activity represents the first evidence of ligand-induced regulation of RPTP activity (Meng et al., 2000). Moreover, an increase in ß-catenin tyrosine phosphorylation was observed as a result of the interaction between pleiotrophin and PTPβ/ζ.

Evidence for a physical interaction between the two molecules was first demonstrated in an investigation for PTPβ/ζ ligands (Maeda et al., 1996). In another case of regulated ligand binding, RPTP activation was demonstrated. The extracellular matrix preparation, matrigel, leads to up-regulation of phoshatase activity of DEP-1 through interactions with the extracellular domain (Sörby et al., 2001).

The extracellular parts of PTPμ, PTPκ and PTPδ interact in a homophilic manner; i.e. the ligands for these receptors are the same proteins expressed on the opposite surface of an adjacent cell (Brady-Kalney et al., 1993; Sap et al., 1994; Wang and Bixby, 1999). In the case of the MAM family of PTPs, the MAM domain serves a critical role in homophilic binding (Zondag et al, 1995). Recently, it has been shown that a small, 11kD extracellular fragment isoform of LAR can also function as a ligand for the same receptor to promote neurite outgrowth (Yang et al., 2003). Components of the extracellular matrix like the laminin-nidogen complex and heparane sulfate proteoglycans are ligands for LAR and PTPσ, respectively. The N-terminal Ig domain of PTPσ and the fibronectin type III domain five of LAR mediate binding to the ligands (Aricescu et al., 2002; O'Grady et al., 1998). The neuronal recognition molecules contactin and TAG/axonin, the adhesion molecules Ng-CAM, N-CAM and Nr-CAM, and the extracellular matrix protein tenascin have been shown to bind to different positions of the extracellar part of PTPβ/ζ (Milev et al., 1994, 1996, 1998; Peles et al., 1995, 1998). However, it has yet to be demonstrated that all these putativ ligands (homophilic or heterophilic) modulate the enzymatic activity of their cognate RPTP in vitro and in vivo.

3.4. Dimerization

Recent data suggest dimerization as an inhibitory mechanism for the regulation of phosphatase activity of RPTP subtype R1/R6 and R4 (Petrone et al., 2000; Majeti and Weiss, 2001). This is in contrast to the RTKs, where dimerization is a well established mechanism for activation. The first data supporting dimerization-induced inactivation was based on a chimeric model system, where EGF-induced dimerization of the EGFR extracellular part induced inactivation of the CD45 intracellular part in chimeric EGFR-CD45 molecules (Desai et al., 1993). Further evidence was obtained when the crystal structure of the membrane proximal catalytic PTP domain of PTPα was solved. The three dimensional structure revealed that the catalytic domain existed as a dimer, in which a helix-turn-helix wedge-shaped

segment of each monomer was inserted into the active site of the other monomer, hence blocking acces to substrate (Bilwes et al., 1996). Mutation in the wedge region in the EGFR-CD45 chimera diminished the EGF-mediated inhibition of PTP activity (Majeti et al., 1998). Consistently, knock-in mice bearing a point mutation in the putative inhibitory wedge of CD45, develop lymphoproliferation and autoimmunity (Majeti et al., 2000). Inhibition of PTPα was shown by introduction of cysteine residues in the extracellular domain. This forced dimerization led to an increase in tyrosine phosphorylation of the PTPα substrate c-src (Jiang et al., 1999). Additionally, the existence of wild-type PTPα homodimers have been demonstrated using cross-linking experiments (Jiang et al., 2000). The study also provided evidence that the homodimerization is due not only to interactions between the catalytic domains, but also involves the extracellular and transmembrane parts. Nevertheless, the presence of transmembrane and extracellular sequences is not required for PTP dimerization.

How dimerization of CD45 and PTPα is modulated is not yet clear since no ligands have been identified for these RPTPs. A recent study revealed that dimerization of CD45 is regulated by the sialylation and O-glycosylation of alternatively spliced CD45 exons in the extrecellular domain (Xu et al., 2002). For example, the smallest CD45 isoform homodimerizes with the highest efficiency, resulting in decreased signaling via the T-cell receptor. In contrast, cysteine oxidation of D2 in PTPα was shown to promote homodimerization of this enzyme (Blanchetot et al., 2002; see below). A cytoplasmic isoform of PTPε (cyt-PTPε), generated by transcription from an alternative promoter, dimerizes and forms higher-order associations in vivo (Toledano-Katchalski et al., 2003). The dimerization state of cyt-PTPε increases by oxidative stress and constitutive formed cyt-PTPε dimers show a markedly reduced PTP activity in vivo and in vitro. PTP domain D2 is involved in intermolecular binding and the presence of D2 in cyt-PTPε results in decreased phosphatase activity.

Dimerization-induced inhibition may, however, not be a general regulatory mechanism for all RPTPs. Analysis of the three-dimensional structure of the membrane proximal catalytic domain of PTPμ revealed a dimeric conformation, but is lacking the interaction between the inhibitory wedge and the active site (Hoffmann et al., 1997). Neither does the structure of the whole cytoplasmic part of LAR indicate that this PTP is inhibited by dimerization (Nam et al., 1999).

RPTP domain D2 has been implicated in the regulation of RPTP activity (see above) and in analogy to D1-D1 homodimerization, D1-D2 heterodimerization has been demonstrated. In a screen for regulatory proteins interacting with domain D1 of PTPσ, domain D2 of PTPδ was identified, but no reciprocal binding was detected. Heterodimeric association between PTPσ-D1 and PTPδ-D1 was shown to result in inhibition of PTPσ-D1s catalytic activity in vitro (Wallace et al., 1998). Direct interactions of several RPTP-D2s, including PTPα-D2, PTPσ-D2, LAR-D2 and PTPμ-D2, with the wedge structure of PTPα D1 have also been reported and a mechanism of cross talk between different RPTPs via D1-D2 interactions has been suggested (Blanchetot et al., 2000). Data supporting the notion that changes in catalytic activity or substrate binding occur remains to be obtained. Yet another mechanism of regulation was demonstrated for PTPμ (Feiken et al., 2000). Here, both catalytic domains are shown to be able to bind the juxtamembrane part of the RPTP. A model is proposed where intramolecular rather than intermolecular interactions between the juxtamembrane region and the two catalytic domains alter the catalytic activity of the phosphatase. In agreement with this, a kinetic study, performed with cytoplasmic PTPμ constructs, pointed out that the in vitro phosphatase activity of PTPμ-D1-D2 is decreased in comparison to PTPμ-D1, indicating negative modulation of PTP activity by D2 via an intramolecular mechanism (Aricescu et al., 2001).

3.5. Phosphorylation

RPTP phosphorylation can result in down- or upregulation of the PTP activity as well as in modulation of the ability to participate in protein-protein interaction. Tyrosine phosphorylation of PTP1B upon association with EGF and insulin receptor has been shown to increase phosphatase activity (Dadke et al., 2001, 2002; Liu and Chernoff, 1997). Similarly, LMW-PTP displays upregulated catalytic activity upon tyrosine phosphorylation by src in response to PDGF stimulation (Bucciantini et al., 1999; Rigacci et al. 1996). Tyrosine phosphorylation of the PTPα carboxy-terminus regulates its binding to Grb2 and thereby modulates its ability to associate with focal adhesion plaques (Den Hertog et al., 1994; Lammers et al., 2000).

Phosphorylation on serine residues has been shown to increase the enzymatic activity of PTPα and to decrease the inhibitory binding to Grb2 (Zheng and Shalloway, 2001). CD45 also displays increased PTP activity upon phosphorylation of serine residues (Wang et al.,

1999). In contrast, PTP-PEST serine phosphorylation results in a decrease in phosphatase activity (Garton and Tonks, 1994).

3.6. Cysteine oxidation

The conserved catalytic cysteine residue in PTPs has a low pK_a due to charge interactions with the microenvironment. The cysteine residue thus exists at physiological pH as a thiolate anion, which is vulnerable to oxidation (Denu and Dixon, 1998; Denu et al., 1995, 1996a, 1996b; Lohse et al., 1997; Zhang and Dixon, 1993). The cysteine can be reversibly oxidized to a sulfenic acid intermediate or further irreversibly oxidized to sulfinic or sulfonic acid forms (Fig. 3.; Denu and Tanner, 1998; Huyer et al., 1997). One of the first evidence of oxidation-mediated inactivation of PTPs was demonstrated upon H_2O_2-treatment of PTP1B and LAR in vitro (Lee et al., 1998). Furthermore, mass spectrometric analysis revealed oxidation-induced glutathionylation of the cysteine (Fig. 3., Barett et al., 1999a; Barret et al., 1999b). This finding supports the notion that glutathionylation is a mechanism to protect the unstable sulfenic acid intermediate from further oxidation to the irreversible sulfinic and sulfonic acid forms. Recently, crystallographic data provided evidence for an alternative mechanism to prevent irreversible oxidation of the catalytic cysteine residue in PTPs. The sulphenic acid intermediate produced in response to PTP1B oxidation is rapidly converted into a previously unknown sulphenyl-amide species, in which the sulphur atom of the catalytic cysteine is covalently linked to the main chain nitrogen of an adjacent residue. Oxidation of PTP1B to the sulphenyl-amide form is accompanied by large conformational changes in the catalytic site that inhibit substrate binding (Salmeen et al., 2003; Van Montfort et al., 2003).

In vivo-oxidation and inactivation of several PTPs including PTP1B, LMW-PTP, SHP1 and SHP2 have been demonstrated in response to H_2O_2-treatment of cells (Chiarugi et al., 2001a, 2001b; Cunnick et al., 1998; Lee et al., 1998; Meng et al., 2002; Xu et al., 2002a). A different mechanism of PTP inactivation has been proposed for PTPα, in which oxidation of a cysteine not involved in catalysis induced stabilization of inhibitory PTPα dimers. The stabilization of the dimeric structure is accompanied by conformational changes of the membrane distal catalytic domain due to oxidation (Blanchetot et al., 2002).

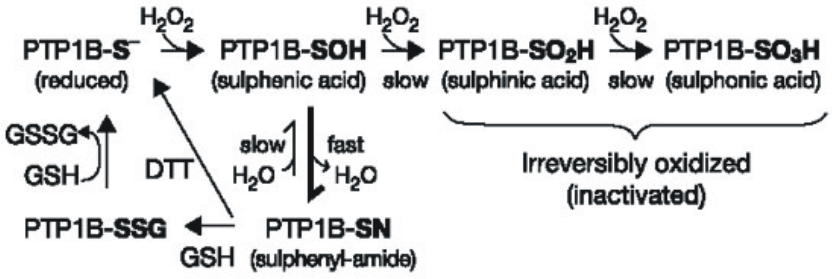

Figure 3: Interrelationship of PTP1B redox species (Salmeen et al., 2003).

The physiological relevance of cysteine oxidation as a regulatory mechanism for PTPs is emphasized by the fact that growth-factor stimulation induces H_2O_2 production, which is crucial for phosphorylation and activation of downstream proteins (Bae et al., 1997; Sundaresan et al., 1995; Rhee et al., 2000). Reduced sensitivity to incorporation of radiolabelled iodacetic acid has been used as readout for active site cysteine oxidation. Stimulation of cells with EGF resulted in a decrease in iodacetic acid bound to PTP1B (Lee at al., 1998). Similar findings have been demonstrated for LMW-PTP upon stimulation of cells with PDGF (Chiarugi et al., 2001a, 2001b). The reversible oxidation was concurrent with a reversible inhibition of PTP activity. Inactivation of PTP1B was observed following stimulation of cells with insulin (Mahadev et al., 2001a, 2001b). PTP inactivation and insulin-induced tyrosine phosphorylation of the insulin receptor were suppressed when cells where pretreated with the H_2O_2 scavenger catalase. SHP2 was identified as a phosphatase undergoing oxidation upon PDGF stimulation of cells and oxidation dependents on association of SHP2 with the PDGF receptor (Meng et al., 2001).

UV-irradiation of cells causes ligand-independend activation of RTKs (Rosette and Karin, 1996; Sachsenmaier et al., 1994) and experimental evidence supports the notion that this is due to UV-inactivation of PTPs. For instance, UV-induced EGFR phosphorylation was linked with a reduced rate in dephosphorylation of the receptor (Knebel et al., 1996). Moreover, inactivation of SHP1, PTPα, PTPδ and DEP-1 has been demonstrated upon UV irradiation of cells (Gross et al., 1999). UV-inhibition of PTP activity can be reversed by addition of reducing agent, indicating oxidation of the active site cysteine as the regulatory mechanism.

4. Biological roles of protein tyrosine phosphatases

Structure and function of PTPs imply physiological significance in the regulation of numerous cellular events. Studies using cell culture as well as transgenic mice indicate that many PTPs are also involved in various growth defects and diseases. Two cellular events in which PTP family members play important roles will be discussed.

4.1. PTPs in cell adhesion

A central mechanism in the regulation of cell-cell and cell-matrix contacts is the tyrosine phosphorylation localized at adherens junctions and focal adhesions. Many of the RPTPs possess structural features resembling classical cell adhesion molecules, suggesting involvment in cell-matrix and cell-cell contacts (Brady-Kalnay and Tonks, 1995).

E-cadherin is a member of a large superfamily of calcium-dependent cell-cell adhesion molecules and comprises the transmembrane component of adherens junctions (AJs) in epithelial tissue. The armadillo family of proteins, ß-catenin or plakoglobin, bind at the E-cadherin C-terminus, and α-catenin links the E-cadherin-armadillo complex to the actin cytoskeleton (Adams and Nelson, 1998; Yap et al., 1997). ß-catenin is a multifunctional protein and it combines the features of a major structural protein at cell-cell junctions with those of a transcriptional co-activator (Barth et al., 1997; Behrens, 1999; Ben-Ze'ev and Geiger, 1998; Bullions and Levine, 1998; Seidensticker and Behrens, 2000). Whereas the great majority of ß-catenin is membrane associated and links the cadherins to actin filaments (Fig. 4, M), the non-junctional ß-catenin is rapidly degraded by the ubiquitin-proteasome system. Stabilization of cytoplasmic ß-catenin (Fig. 4, C) by wnt signaling leads to its nuclear accumulation (Fig. 4, N), complexing with LEF/TCF transcription factors, and to transactivation of LEF/TCF target genes (Eastman and Grosschedl, 1999; Nusse, 1999; Roose and Clevers, 1999).

Both, cadherin-mediated adhesion as well as nuclear signaling by ß-catenin play important roles in differentiation, development, and cancer initiation/progression. In vertebrate development and mesoderm formation, the regulated loss of the E-cadherin-mediated adhesion system can initiate epithelial-mesenchymal transitions; in tumors, this process can result in rapid progression of relatively benign adenomas to invasive, metastatic carcinomas

(Daniel et al., 1997; Hazan et al., 1998; Gumbiner, 1997; Graff et al., 1998; Roura et al., 1999; Tsukamoto et al., 1999; Lilien et al., 2001; Piedra et al., 2001). In contrast, nuclear signaling by ß-catenin is involved in the regulation of cell fate during embryonic development (Wodarz and Nusse, 1998), and aberrant activation of ß-catenin-mediated transactivation might contribute to cancer initiation by causing increased cell proliferation (Ben-Ze'ev, 1997; Ben-Ze'ev and Geiger, 1998; Morin, 1999; Polakis, 1999).

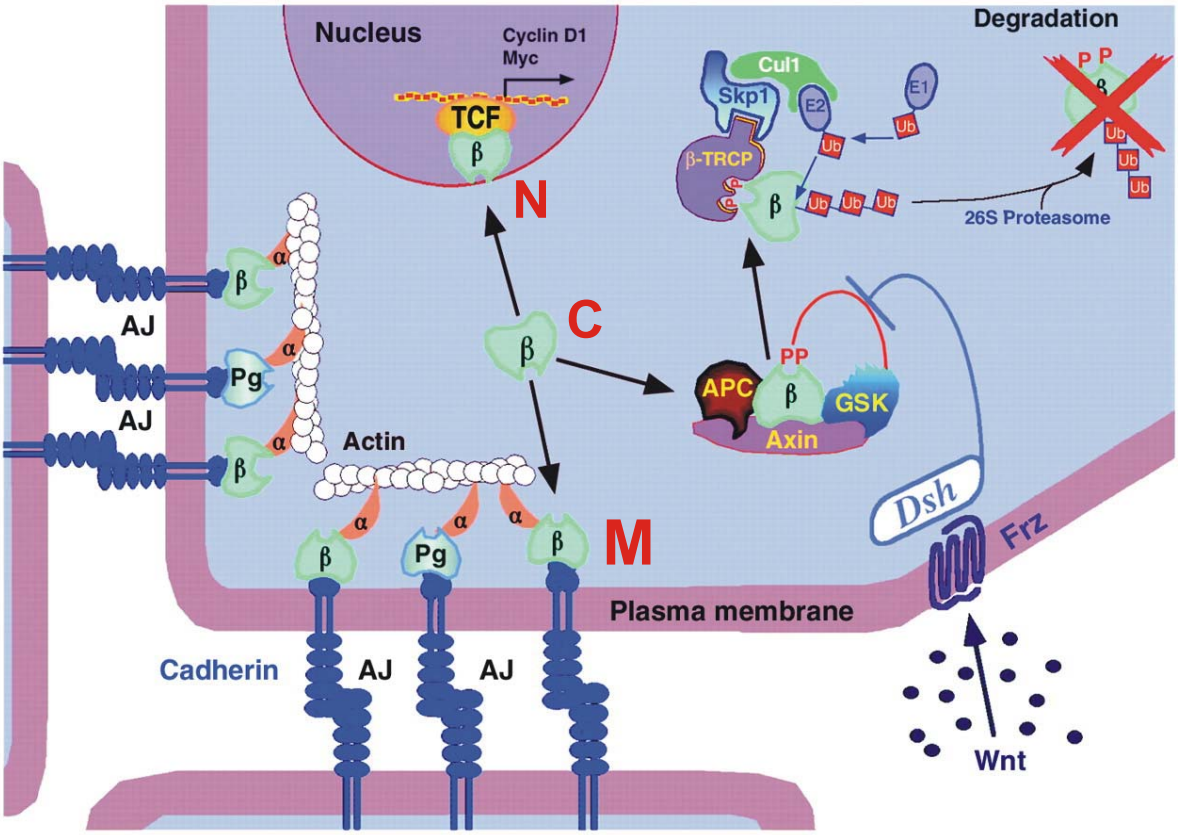

Figure 4: Interactions involving membrane-associated (M), cytoplasmic (C) and nuclear (N) ß-catenin. *See text for further information. Abbreviations: α-catenin (α), ß-catenin (ß), plakoglobin (Pg), adherens junctions (AJ), glycogen synthase kinase (GSK), ubiquitin (Ub), frizzled receptor (Frz), disheveled (Dsh)(Zhurinsky et al., 2000).*

E-cadherin is a classical tumor suppressor protein and E-cadherin-mediated cell adhesion in cancer cells is inactivated by multiple mechanisms (Guilford et al., 1998; Gayther et al., 1998; Vleminckx et al., 1991; Mareel et al., 1995; Perl et al., 1998; Vos et al., 1997; Muta et al., 1996; Ilyas et al., 1996; Berx et al., 1995, 1996, 1998; D'Souza et al., 1994; Yoshiura et al., 1995; Graff et al., 1998; Guilford, 1999; Voeller et al., 1998; Beavon et al., 1999, 2000; Christofori et al., 1999). Tyrosine phosphorylation of ß-catenin is one such mechanism. Enhanced phosphorylation of tyrosine residues on ß-catenin by the EGFR, ErbB2, c-met and

src is almost invariably associated with a decrease in the amount of membrane-associated ß-catenin, an increase in the cytoplasmic "pool" of ß-catenin, and consequently, disruption of the cadherin-actin connection concomitant with loss of adhesive function (Hoschuetzky et al., 1994; Hirohashi et al., 1998; Shiozaki et al., 1995; Efstathiou et al., 1998; Takahashi et al. 1997, Jawhari et al., 1999; Ochiai et al., 1994; Kanai et al., 1995; Hiscox et al., 1999a, 1999b). Recently, tyrosine phosphorylation of ß-catenin has been reported to increase the interaction of ß-catenin with the TATA-box binding protein (TBP), which results in increased transcriptional activity of the ß-catenin/Tcf complex (Piedra et al., 2001). However, there is some evidence that cells with non-functional cadherin-catenin complexes do not display an increased ß-catenin-mediated transcriptional activity (van de Wetering et al., 2001).

Several PTPs have the potential to alter the state of phosphorylation of the cadherin-catenin complex. Members of four distinct PTP families have been reported to alter either ß-catenin phosphorylation and/or be correlated with the state of phosphorylation of cadherin itself (Tab. 1). Evidence suggests that PTP1B maintains ß-catenin in a dephosphorylated state. For instance, introduction of a dominant-negative construct of PTP1B in cells results in hyper-phosphorylation of ß-catenin, an increase in the cytoplasmic pool of tyrosine phosphorylated ß-catenin, and dissociation of the cadherin-actin connection, concomitant with loss of cadherin function (Balsamo et al., 1996, 1998). This dominant-negative PTP1B construct also inhibits neurite extension by PC12 cells on N-cadherin substrates, as does downregulation of PTP1B by means of antisense oligonucleotides (Pathre et al., 2001). Consistent with these observation, PTP1B is present at adherens junctions and localizes to growth cones (Balsamo et al., 1998; Pathre et al., 2001). PTP1B associates directly with the cytoplasmic domain of N-cadherin, as does the dominant-negative construct (Balsamo et al., 1996, 1998; Rhee et al., 2001; Xu et al., 2002c). The targeted site in N-cadherin partially overlaps with the ß-catenin-binding site but there is no competition between PTP1B and ß-catenin for binding to the N-cadherin cytoplasmic region (Xu et al., 2002c). Interestingly, the interaction between PTP1B and N-cadherin depends on phosphorylation of tyrosine 152 in PTP1B (Rhee et al., 2001).

LAR has been shown to interact with and dephosphorylate ß-catenin (Kypta et al., 1996). Additionally, overexpression of LAR correlates with prevention of ß-catenin tyrosine phosphorylation and inhibition of epithelial cell migration (Müller et al., 1999). Similarly, PTPβ/ζ interacts with and dephosphorylates ß-catenin. Furthermore, interaction of

PTPβ/ζ with its ligand, pleiotrophin, results in inactivation of its intrinsic catalytic activity and enhanced tyrosine phosphorylation of ß-catenin (Meng et al., 2000). The MAM-family PTPs PTPκ (Fuchs et al., 1996), PTPρ (Cheng et al., 1997) and PCP-2 (Yan et al., 2002) interact directly with ß-catenin and PTPκ as well as PCP-2 have actually been shown to dephosphorylate ß-catenin. The interaction between PTPκ/PCP-2 and ß-catenin requires the juxtamembrane cadherin-like sequence of the enzymes.

PTP	BINDING PROTEIN	SUBSTRATE PROTEIN	BIOLOGICAL CONSEQUENCES	REFERENCES
PTP1B	N-cadherin	ß-catenin	Stabilization of the cadherin-actin connection Strengthen of adhesive function Inhibition of neuronal outgrowth	Balsamo et al., 1996, 1998 Rhee et al., 2001 Pathre et al., 2001 Xu et al., 2002
LAR	ß-catenin	ß-catenin	Inhibition of epithelial cell migration	Kypta et al., 1996 Müller et al., 1999
PTPμ	N-cadherin $P120^{ctn}$	N-cadherin $P120^{ctn}$	Inhibition of neuronal outgrowth	Brady-Kalnay et al., 1998 Burden-Gulley et al., 1999 Zondag et al., 2000
PTPκ	ß-catenin	ß-catenin	n.d.	Fuchs et al., 1996
PTPρ	ß-catenin	n.d.	n.d.	Cheng et al., 1997
PTPβ/ζ	ß-catenin	ß-catenin	n.d.	Meng et al., 2000
PCP-2	ß-catenin	ß-catenin	Inhibition of cell migration	Yan et al., 2002
PTP-Pez	ß-catenin	ß-catenin	Inhibition of cell motolity	Wadham et al., 2003

Table 1: PTPs involved in regulation of the cadherin-catenin adhesion complex. *Both, binding and substrate proteins are constituents of the AJs. n.d.- not determined.*

In contrast to its close relatives, PTPμ interacts with and potentially dephosphorylates cadherin (Brady-Kalnay et al., 1995, 1998). PTPμ binds directly to the carboxy-terminal 38 amino acids of E-cadherin, a region that includes most of the minimal PTP1B binding site. This might suggest that the interaction of PTPμ and PTP1B are mutually exclusive and may, under certain circumstances, play the same role, maintaining the stability of cadherin adhesions. This is further suggested by the fact that downregulation of either PTP1B (Pathre et al., 2001) or PTPμ (Burden-Gulley and Brady-Kalnay, 1999) mediated by antisense oligonucleotides or a dominant-negative construct, blocks N-cadherin-mediated neuronal outgrowth. The absence of PTPμ is correlated with increased phosphorylation of cadherin itself, not ß-catenin (Brady-Kalnay et al., 1998). Since increased tyrosine phosphorylation of

N-cadherin has been associated with increased turnover of N-cadherin through cleavage and release of a 90 kD extracellular fragment (Lee et al., 1997), PTPμ may regulate cadherin turnover and therefore, the relative amount at the cell surface. A further possibility is that PTPμ alters the affinity of $p120^{ctn}$ for cadherin through dephosphorylation (Zondag et al., 2000) and that this affects cadherin-mediated adhesion.

In neuronal circuits synapses correspond to AJs in epithelial cell layers and the cadherin-catenin complex facilitates the formation and maintainance of synaptic junctions, coordinates pre- and postsynaptic structural changes via links to the actin cytoskeleton, and furthermore, modulates the adhesiveness of synaptic junctions according to the state of pre- and postsynaptic terminals (Ranscht, 2000; Goda, 2002; Togashi et al., 2002). Recently, it was demonstrated that membrane depolarization causes an increase in synaptic ß-catenin concomitant with an increase in the cadherin-ß-catenin association. Moreover, tyrosine kinase inhibitors promoted ß-catenin redistribution into the dendritic shafts. Mutation of a tyrosine residue to phenylalanine in ß-catenin caused its accumulation in synapses, whereas a phosphorylation-mimic ß-catenin mutant localized in the dendritic shafts (Murase et al., 2002).

Like adherens junctions, focal adhesions (FAs) are crucial for the maintainance of the epithelial tissue structure and integrins are the cell adhesion receptors which connect the extracellular matrix proteins, such as fibronectin, vitronectin, collagens and laminins, to intracellular protein complexes in contact with the actin cytoskeleton. These focal contacts represent also one of the major sites of tyrosine phosphorylation within the cell. Proteins in these complexes include focal adhesion kinase (FAK), paxillin, tensin, talin, vinculin, α-actinin, src, cortactin and $p130^{cas}$ (Giancotti and Ruoslahti, 1999). FAs are dynamic structures that assemble, disperse and recycle as cells migrate or enter into mitosis. Increased tyrosine phosphorylation accompanies FA disruption (Crowley et al., 1995) and src family kinases (Klinghoffer et al., 1999; Fincham et al., 1998) and FAK (Ilic et al., 1995; Cary et al., 1996) have been implicated in the regulation of FA disassembly and turnover.

Significant progress has also been made in identifying PTPs that are involved in the regulation of FA assembly or disassembly. PTP1B was shown to bind to, and to dephosphorylate, $p130^{cas}$ and overexpression of PTP1B, but not of a proline-to-alanine mutant form (PA-PTP1B), that is unable to bind or dephosphorylate $p130^{cas}$, interfered with cell spreading, cytoskeletal architecture, and the formation of focal adhesion complexes. Cells

overexpressing wild-type PTP1B also displayed markedly reduced migration, whereas cells expressing the PA-PTP1B mutant migrated normally (Liu et al., 1998). Recently, an interaction between PTP-PEST and paxillin was demonstrated and the LIM motifs of paxillin were shown to be crucial for PTP-PEST binding (Brown et al., 2002). Knockout cell lines of either SHP-2 or PTP-PEST exhibit enhanced FAs (Yu et al., 1998; Angers-Lousteau et al., 1999a, 1999b). In addition, two transmembrane PTPs, LAR and PTPα, have been shown to localize in FAs under restricted conditions (Serra-Pages et al., 1995; Lammers et al., 2000).

4.2. PTPs that antagonize growth factor-induced signaling

Different approaches have been used, aiming at identifying PTPs that antagonaize RPTK-induced signaling cascades. These include overexpression of PTPs, use of substrate trapping mutants, knock-out studies and screenings using libraries of PTPs. Below is discussed the involvement of PTPs in two growth factor-induced signaling cascades: EGF and FGF receptor-initiated signaling.

PTPs are highly specific enzymes, dephosphorylating not only specific protein substrates within a cellular context, but also specific phospho-tyrosine residues within any one given substrate (Tonks and Neel, 2001; Tiganis et al., 1998; O'Reilly and Neel, 1998). For instance, at least five major phospho-tyrosine residues have been identified in the EGFR, and taken the different tissues into account, in which the EGFR is expressed, it is probable that numerous PTPs participate in EGFR dephosphorylation (Tiganis, 2002). SHP-1 associates via its N-terminal SH2 domain with pTyr-1173 on the EGFR and inhibits EGF-induced activation of the receptor (Tenev et al., 1997; Keilhack et al., 1998). SHP-1 may also play an important role in GPCR-mediated inhibition of EGFR signaling (Shibasaki et al., 2001). Substrate trapping mutants of PTP1B and TC-PTP have both been shown to form complexes with the EGFR (Flint et al., 1997; Tiganis et al., 1998). EGFR activation can cause accumulation of TC-PTP (TC48 isoform) substrate trapping mutant in the cytoplasm where it forms stable complexes with the tyrosine phophorylated EGFR (Tiganis et al., 1999). PTP1B-deficient fibroblasts exhibit enhanced EGFR tyrosine phosphorylation with little or no hyperactivation of the downstream ERK2 and Akt pathways (Tonks and Neel, 2001).

Antisense experiments have revealed that both LAR and PTPδ are involved in dephosphorylation of the EGFR (Kulas et al., 1996; Pestana et al., 1999; Fig. 5). Additionally, the ganglioside GM3 was reported to stimulate PTPδ activity to dephosphorylate the EGFR and cells became refractory when PTPδ expression was inhibited by antisense strategies

(Pestana et al., 1997). LAR and PTPδ may play a significant role in GPCR-induced EGFR inhibition. For instance, bradykinin decreases basal and ligand-induced EGFR tyrosine phosphorylation and bradykinin stimulation of cells leads to increased PTP activity of LAR and PTPδ in cell lysates (Graness et al., 2000).

Upon cell-cell contact formation, proliferation of cells is inhibited ("contact inhibition") and it has been suggested that PTPs, by suppressing RTK signaling, may play a key role in this process. Membrane-associated PTP activity and/or RPTP expression are elevated when cells become confluent (Pallen et al., 1991; Sörby et al., 1996; Ostman et al., 1994; Fuchs et al., 1996; Gebbink et al., 1995; Bianchi et al., 1999; Symons et al., 2002). Moreover, PTP inhibitors can induce contact-inhibited cells to re-enter the cell cycle (Klarlund, 1985; Suzuki et al., 2000), indicating that PTP catalytic activity is essential for cell contact-mediated growth inhibition. Interestingly, the MAM-family of PTPs (PTPκ, PTPμ), PTPδ and a splice isoform of LAR can function as homophilic cell adhesion receptors, suggesting that these enzymes may play a role in signaling initiated by the formation of cell-cell contacts. EGFR activation and signaling are inhibited in dense cell cultures and this correlates with enhanced PTP-activity (Sörby et al., 1996). Expression of both LAR and PTPδ can be elevated in dense compared to sparse cell cultures (Bleyle et al., 1999; Celler et al., 1995., Symons et al., 2002), suggesting that both PTPs could contribute to contact-mediated inhibition of EGFR signaling.

It is well accepted that different growth factors induce the tyrosine phosphorylation of distinct sets of proteins and the formation of distinct signaling complexes. For example, FGF induces phosphorylation of FRS2, whereas EGF induces the phosphorylation of SHC (Kouhara et al., 1997; Xu et al., 1998; Carpenter and Cohen, 1990; Boonstra et al., 1995; Wells et al., 1999). These differences define the divergent biological responses induced by different growth factors. But a single growth factor can also activate different signaling pathways and biological responses in different cell types, or in the same cell type but under different conditions. The precise regulatory mechanisms that underlie the initial tyrosine phosphorylation reactions and the formation of signaling complexes, especially the role PTPs in such mechanisms, are not well characterized.

One of the key signaling complexes recruited to the plasma membrane by tyrosine phosphorylated proteins in response to growth factor stimulation comprises GRB2 and SOS1. The recruitment of the GRB2-SOS1 complex brings it into close contact with RAS, which it activates by catalysing the conversion of GDP-bound RAS to the GTP-bound form. Activated

RAS in turn induces a cascade of phosphorylation events that result in the activation of MAPK (Ahn, 1993; Seger and Krebs, 1995; Cobb, 1999; Marshall, 1995).

PTPs involved in FGFR dephosphorylation have not been described, but the function of LAR in FGF signaling has been examined. Cells stably expressing LAR showed a decreased MAPK phosphorylation upon FGF, but not EGF stimulation. Importantly, LAR did not affect the autophosphorylation of the growth factor receptors. The specific effect of LAR on FGF-induced MAPK activation seems to be mediated by specific dephosphorylation of FRS2 and thus prevention of the interaction between FRS2 and GRB2 (Fig. 5). In contrast, LAR selectively inhibited the EGF-induced phosphorylation of $p130^{cas}$ and the formation of the $p130^{cas}$/GRB2 complex, but this effect did not influence the activation of MAPK by EGF (Wang et al., 2000). These data suggest that LAR and similar RPTPs may contribute to the regulation of growth factor-induced signaling by selectively inhibiting the tyrosine phosphorylation of specific signal transducers that act downstream of the receptors and that RPTPs may exert distinct effects on signaling complex formation induced by the different growth factor receptors.

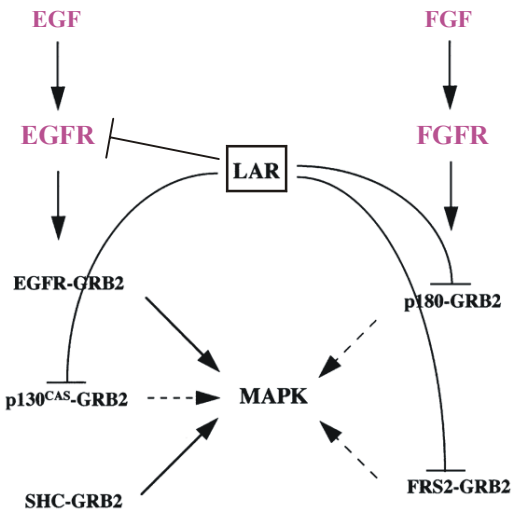

Figure 5: Proposed model for the role of LAR in EGF and FGF receptor signaling.

5. Limited proteolysis of type I membrane-spanning proteins

Proteolytic enzymes are involved in a great variety of physiological processes and their action can be divided into two different categories: (I) limited proteolysis, in which a protease cleaves only one or a limited number of peptide bonds of a target protein leading to its activation or maturation and (II) protein degradation, in which proteins are cleavad into their amino acid constituents. The proteins to be degraded are usually first conjugated to multiple molecules of the polypeptide ubiquitin. This modification marks them for rapid hydrolysis by the proteasome in the presence of ATP. Another pathway consists in the compartmentation of proteases in lysosomes.

Proteinases are classified according to their catalytic mechanisms into four different classes: serine, cysteine, aspartic and metallo (zinc) proteases. Exemplarily, some members of each family that directly act on type I membrane-spanning proteins will be discussed.

5.1. Processing by proprotein convertases (PCs)

Type I transmembrane proteins do by definition span the cell membrane bilayer once, oriented with their NH_2 termini in the lumen and their COOH termini in the cytosol. In many cases these proteins are synthesized as high-molecular-mass precursors that are proteolytically converted by proprotein convertases (PCs) into smaller fragments during the course of secretion to the cell surface. Examination of the primary sequences of substrate proteins revealed that the cleavage site is characterized by the presence of paired basic residues, usually R/K-R or R-X-X-R, where X can be any amino acid.

PCs constitute a seven-member family of serine endoproteases in mammalian species (Tab. 2), including furin, PC1/3, PC2, PC4, PACE4, PC5/6 and LPC/PC7/PC8/SPC7 (Nakayama, 1997; Taylor et al., 2003; Zhou et al., 1999; Seidah et al., 1999 and Steiner et al., 1998). On the basis of their tissue distribution, three groups of PCs can be differentiated. Furin, PACE4, PC5/6 and LPC are expressed in a broad range of tissues and cell lines. In contrast, expression of PC2 and PC1/3 is restricted to neuroendocrine tissue, whereas expression of PC4 is limited to testicular spermatogenic cells 1992) (Tab. 2).

PC	Tissue distribution	Cellular compartment	Null phenotype
Furin	Ubiquitous	TGN, endosomal	Embryonic lethality (day 10.5), ventral closure defects, impaired axial rotation
PC1/3	Neuroendocrine	Secretory granules	Mouse: severe growth defects, defective GHRH and POMC, hyperinsulineamia Humans[1]: severe early obesity, adrenocortical insufficiency, hyperinsulineamia
PC2	Neuroendocrine	Secretory granules	Retarded growth post partum, hypoglycaemia, proinsulinaemia, glucagon deficiency, opiod peptide processing defects
PC4	Germ cells	ND	Reduced fertility
PACE4	Ubiquitous	TGN, endosomal	Embryonic lethality (day 15.5.), craniofacial and CNS defects
PC5/6	Ubiquitous	TGN, endosomal, secretory granules	ND
LPC	Ubiquitous	TGN, endosomal system, cell surface	Viable, misexpression causes thymic defects

Table 2: The proprotein convertase family. *Included are the phenotypes of the PC null models available and the PC1 null patients[1]. CNS, central nervous system; GHRH, growth hormone-releasing hormone; ND, not determined; POMC, proopiomelanocortin; TGN, trans-Golgi network.*

5.2. Shedding by ADAMs

The extracellular fragments of several integral membrane proteins are released from the cell surface, often in a fully functional form, by transmembrane zinc proteases known as "sheddases or secretases" through a process called "ectodomain shedding". ADAMs (a disintegrin and metalloprotease) have been implicated in most of the known shedding events (Seals and Courtneidge, 2003; Black and White, 1998; Schlondorff and Blobel, 1999; Primakoff and Myles, 2000; Kheradmand and Werb, 2002). For instance, cleavage and shedding of cytokine receptors, growth factor precursors and their receptors, amyloid precursor protein, prions, cell adhesion molecules, Notch and its ligand, delta, are mediated by ADAMs. The cleavage site is generally located close to the membrane surface and it is thought that cleavage depends upon access to the stalk region of the substrate protein. Thus, the topology of the substrate membrane protein defines that only certain cell-surface proteins will be susceptible to release (Hooper et al., 1997).

In humans there are 19 adam genes, as shown in Tab. 3. This family is often also referred to as the MDC family, indicating the presence of metalloprotease, disintegrin and cysteine-rich domains. The most well-studied member is TNFα converting enzyme (TACE) or ADAM17 (Black, 2002). However, processing of the TNFα precursor can still occur in cells derived from ADAM17-deficient mice, indicating that other ADAM family members may

ADAM	Common name(s)	Potential functions	Expression	Alternative splicing	Domain function: MP active	Domain function: Integrin binding	Domain function: PxxP
2	fertilin-β, PH-30β	sperm/egg binding/fusion	testis[1]			√	
7	EAP1		epididymis[2]				√
8	MS2, CD156		granulocytes/ monocytes[3]		√(d)		√
9	meltrin-γ, MDC9	sheddase, cell migration	somatic[4]	√(FL,s)[5]	√(d)	√	√
10	Kuz, MADM, SUP-17	sheddase; cell fate determination	somatic[6]	√(L,S)[7]	√(d)		√
11	MDC	putative tumor repressor	brain[8]	√[9,10]			
12	meltrin-α	sheddase, myoblast fusion	somatic[11,12,13]	√(L,S)[13]	√(d)	√[a]	√
15	metargidin, MDC15	cell/cell binding	somatic[14]		√(p)	√	√
17	TACE	sheddase	somatic[15]		√(d)		√
18	tMDCIII		testis[16]				
19	meltrin-β, MADDAM	sheddase, dendritic cell dev.	somatic[11,17]		√(d)		√
20			testis[18]		√(p)		
21			testis[18]		√(p)		
22	MDC2		brain[8,19]	√(γ,δ,ε)[19]			√
23	MDC3	cell adhesion/neural dev.	brain[8,20]			√	
28	MDC-L	immune surveillance	epididymis, lung, lymphocytes[21,22,23]	√(ms,)[21]	√(d)	√	
29			testis[24,25]	√(α,β,γ)[24]			√
30			testis[24]	√(α,β)[24]	√(p)		
33		genetically linked to asthsma	somatic[26]	√	√(p)		√

Table 3: Human ADAMs.

[1] (Gupta et al. 1996) [2](Lin et al. 2001) [3](Yoshiyama et al. 1997) [4](Weskamp et al. 2002) [5](Hotoda et al. 2002) [6](Chantry and Glynn 1990) [7](Yavari et al. 1998) [8](Sagane et al. 1998) [9](Katagiri et al. 1995) [10](Wu et al. 1997) [11](Yagami-Hiromasa et al. 1995) [12](Harris et al. 1997) [13](Gilpin et al. 1998) [14](Kratzschmar et al. 1996) [15](Patel et al. 1998) [16](Frayne et al. 2002) [17](Kurisaki et al. 1998) [18](Poindexter et al. 1999) [19](Harada et al. 2000) [20](Cal et al. 2000) [21](Roberts et al. 1999) [22](Howard et al. 2000) [23](Howard et al. 2001) [24](Cerretti et al. 1999) [25](Xu et al. 1999) [26](Yoshinaka et al. 2002)

MP Active refers to either predicted (p) or demonstrated/ published (d) activity based on the amino acid sequence of the catalytic active site.

[a] Reference to the syndecan-binding activity of ADAM12's cytsteine-rich domain (see text).

PxxP refers to the presence of SH3-binding sites in cytoplasmic tail domains.

process TNFα as well (Reddy et al., 2000). ADAM9, 17, 10 and 12 have been implicated in the release of soluble HB-EGF, that in turn activates the EGF receptor (Izumi et al., 1998; Yan et al., 2002; Hart et al., 2004; Sunnarborg et al., 2002; Asakura et al., 2002). ADAM17 has also been shown to process HER4/erbB4 (Rio et al., 2000). Processing of the amyloid precursor protein (APP) is mediated by ADAM10 and 17, but it has been suggested that individual proteases regulate APP shedding independently (Slack et al., 2001). ADAM10 has been implicated in cleavage of Notch and its ligand delta (Lieber et al., 2002). In this scenario, ADAM-mediated cleavage of Notch is induced upon binding to delta and genetic analysis demonstrates that either loss of function or depletion mutants in ADAM10 exhibit similar phenotypes to loss-of-function mutations in Notch (Rooke et al., 1996; Wen et al., 1997; Hartmann et al., 2002). Moreover, Ephrin-A2 cleavage triggered by Eph receptor binding was shown to depend on ADAM10 and suggested to result in contact-mediated axon repulsion (Hattori et al., 2000).

5.3. Regulated intramembrane proteolysis (Rip) by Presenilins

The realization that cell surface receptors can be cleaved within the membrane bilayer to liberate cytosolic fragments that enter the nucleus to control gene transcription has opened up a new field in cell signaling. Such a route of information transfer from the extracellular environment to the nucleus is not dependent on cytoplasmic signaling networks that conduct and amplify the signal by means of enzymes, second messengers and adapator proteins (Brown et al., 2000).

Regulated intramembrane proteolysis of type I transmembrane receptors is generally performed by presenilin proteases, also called γ-secretases. Presenilins are unique aspartic proteases that contain eight transmembrane domains and that are activated by autoproteolysis. Presenilins act as heterodimers, which are thought to combine with additional integral membrane proteins such as nicastrin and aph-1 to form a large active protease complex.

Table 4 lists proteins that are known or postulated to undergo Rip via presenilin-mediated processing. For three of the proteins (Notch, N-Cadherin and Ire1), the functions of their released cytoplasmic fragments, that accumulate in the nucleus, have been identified (Tab. 4). For instance, the Notch fragment binds to the transcription factor CBF1, thereby activating gene transcrition. In contrast, the N-Cadherin cytoplasmic portion induces degradation of the transcriptional coactivator CBP, thereby inhibiting gene transcription. Intriguingly, the

cytoplasmic fragment of Ire1 consists of an endonuclease domain, that, once in the nucleus, induces splicing of a transcription factor coding mRNA and thereby activates gene transcription.

Protein	Function of membrane-released proteolytic products	Reference
Notch	Activation of gene transcription by binding to transcription factors of the CSL family	Baron, 2003 Selkoe and Kopan, 2003
APP	Neurotoxic, causes neurodegeneration and Alzheimer's disease	De Strooper et al., 1998, 1999 Wolfe et al., 1999
ErbB4	Unknown	Ni et al., 2001
E-Cadherin	Disassembly of adherent junctions	Marambaud et al., 2002
N-Cadherin	Promotes degradation of the transcriptional coactivator CBP	Marambaud et al., 2003
CD44	Unkown	Lammich et al., 2002
Ire1	Unfolded protein response	Niwa et al., 1999
LRP	Unknown	May et al., 2002

Table 4: Transmembrane proteins that undergo Rip in mammalian cells. *All proteins are targeted by presenilins.*

The mechanism of proteolytic processing of Notch has been investigated in detail (Fig. 6). Notch is cleaved by three proteases at three different sites: cleavage on site one (S1 cleavage) is performed by furin, cleavage at S2 is mediated by ADAM10 and 17 (S2 cleavage), followed by presenilin-catalysed processing at site 3 (S3 cleavage). Importantly, the release of the cytosolic tail of Notch by S3-processing does not take place until the extracellular bulk of the protein has been removed by ADAM-mediated S2 cleavage, explaining the fact of why metalloprotease inhibitor treatment diminished Rip.

5.4. Cleavage by cytoplasmic proteases

Two protease families have been mainly implicated in cleavage of type I transmembrane proteins in the cytosol: caspases and calpains. Caspases are cysteine proteases which specifically cleave their substrates after aspartate residues. 11 caspases have been identified in humans. After initiation of apoptosis these enzymes become processed and activated to directly execute programmed cell death by the specific cleavage of substrate proteins. For instance, E-Cadherin is targeted by caspases following staurosporine treatment within the juxtamembrane region, leading to disruption of cell-cell contacts (Steinhusen et al., 2001).

Calpains are cysteine proteases and two family members, µ-Calpain and m-Calpain, have been isolated as proteins. Both are specifically activated by micromolar concentrations of free calcium and both function as heterodimers. Key signal transduction molecules have actually been shown to be cleavaed by calpains and consequently, calpains have been implicated in

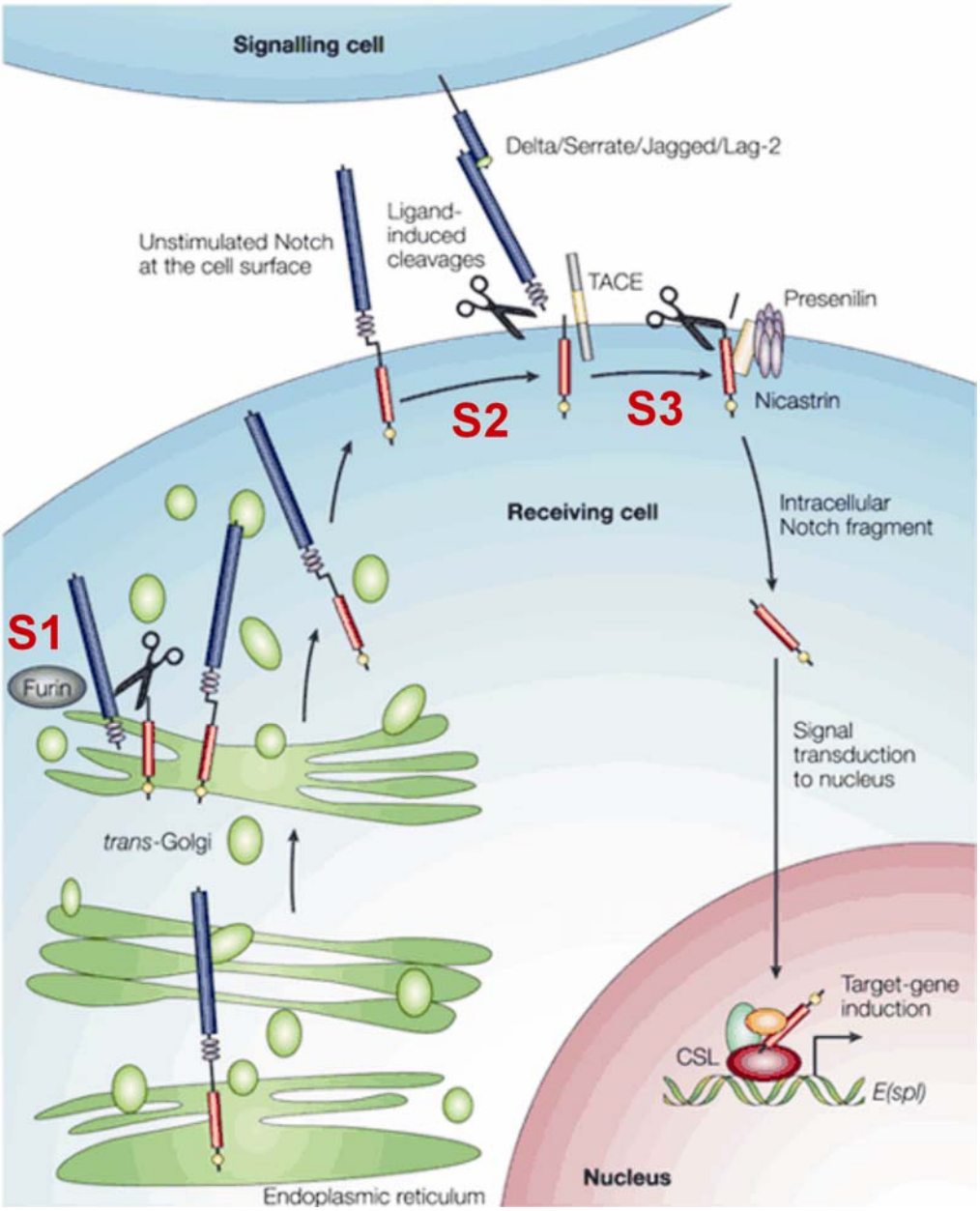

Figure 6: Scheme depicting S1, S2 and S3 processing as proteolytic regulatory mechanism of Notch. *Notch is thought to be cleaved on three sites: S1, S2 and S3. S1 cleavage occurs within the secretory pathway so that a processed heterodimeric form is transported to the cell surface. TACE or KUZ-mediated S2 cleavage occurs following ligand binding by Delta/Serrate/Jagged and generates a membrane-tethered form. The latter is a constitutive substrate for S3 cleavage, which releases the soluble intracellular domain of Notch. The S3 product is translocated to the nucleus where it binds to a transcription factor, Suppressor of hairless/CBF1, thereby activating gene transcription.*

regulation of the cell cycle, cell migration and adhesion, apoptosis, long-term-potentiation and cancer (Perrin and Huttenlocher, 2002). The EGFR is targeted by calpains and cleavage of PTPα/ε results in translocation of the enzyme into the cytoplasm, reducing its ability to act on molecules located at the cell membrane (Gil-Henn et al., 2001).

II. Specific Aims

The main purpose of this thesis was to investigate the mechanism of regulation of the Receptor-like Protein Tyrosine Phosphatase κ with the object to understand of how signaling of the protein is initiated and of whether this process is disturbed in cancer cells. In particular, we sought to explore the mechanisms leading to generation of several to date undescribed κ isoforms.

Another aim was to raise an antidody recognizing κ's extracellular portion, with the potential to modulate κ's activity and signaling in order to use it as a tool for substrate identification and functional analysis.

The third purpose was to screen for and to identify soluble, extracellular RPTPκ-binding proteins (putative RPTP ligands).

III. Materials and Methods

1. Material sources

1.1. Laboratory chemicals and biochemicals

Acrylamide	Serva, Heidelberg
Agar	Difco, USA
Agarose	BRL, Eggenstein
Ampicillin	Roche, Mannheim
Aprotinin	Sigma, Taufkirchen
APS (Ammonium peroxodisulfate)	Bio-Rad, München
ATP (Adenosine triphosphate)	Amersham Pharmacia, Freiburg
[γ-^{32}P] ATP (>5000 Ci/mmol)	Perkin Elmer/NEN, Köln
BAPTA	Sigma, Taufkirchen
BAPTA-AM	Sigma, Taufkirchen
Batimastat	British Biotech, UK
Bisacrylamide	Roth, Karlsruhe
Bromphenol blue	Sigma, Taufkirchen
BSA (Bovine serum albumin)	Sigma, Taufkirchen
Coomassie G250	Serva, Heidelberg
Chloroquin	Biotrend Chemikalien, Köln
Chlorpromazine	Sigma, Taufkirchen
Crystal Violet	Sigma, Taufkirchen
Deoxynucleotides (dG/A/T/CTP)	Roche, Mannheim
DTT (Dithiothreitol)	Sigma, Taufkirchen
E64d	Sigma, Taufkirchen
EGF	Sigma, Taufkirchen
Ethidium bromide	Sigma, Taufkirchen
Fluophenazine	Sigma, Taufkirchen
Fibronectin	Calbiochem, Bad Soden
Formaldehyde	PolySciences, Eppenstein
Geneticin (G418, GibCo)	Invitrogen, Eggenstein
GF109203X	Sigma, Taufkirchen
HEPES (N-(2-Hydroxyethyl)piperazine-N`-(2-ethanesulfonic acid))	Serva, Heidelberg
Ionomycin	Sigma, Taufkirchen
IPTG (Isopropyl β-D-1-thiogalactopyranoside)	Biomol, Hamburg
Lactacystin	Sigma, Taufkirchen
L-Glutamine (GibCo)	Invitrogen, Eggenstein
Leupeptin	Sigma, Taufkirchen
Lipofectamine® (GibCo)	Invitrogen, Eggenstein
Lysozyme	Sigma, Taufkirchen
MBP (Myelin basic protein)	Sigma, Taufkirchen
Methyl-ß-cyclodextrin	Sigma, Taufkirchen
MG132	Sigma, Taufkirchen
Plasminogen (from human plasma)	Sigma, Taufkirchen
PMSF (Phenylmethanesulfonyl fluoride)	Sigma, Taufkirchen

PMA	Sigma, Taufkirchen
pNPP (p-Nitrophenyl phosphate)	Sigma, Taufkirchen
Polybrene (Hexadimethrine bromide)	Sigma, Taufkirchen
Polyfect®	Quiagen, Hilden
PD98059	Alexis, Grünberg
Ponceau S	Sigma, Taufkirchen
Promazine	Sigma, Taufkirchen
SDS (Sodium dodecyl sulfate)	Roth, Karlsruhe
Sodium azide	Serva, Heidelberg
Sodium fluoride	Sigma, Taufkirchen
Sodium orthovanadate	Sigma, Taufkirchen
Superfect®	Quiagen, Hilden
T4 gene 32 protein	Pharmacia, Freiburg
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Serva, Heidelberg
TPA (Tetradecanoyl-phorbol-13-acetate)	Sigma, Taufkirchen
Trifluoperazine	Sigma, Taufkirchen
Triton X-100	Serva, Heidelberg
Tween 20, 40	Sigma, Taufkirchen
Tyrphostin AG1478	Alexis, Grünberg
U0126	Calbiochem, Bad Soden

All other chemicals were purchased in analytical grade from Merck (Darmstadt).

1.2. Enzymes

Calf Intestine Alkaline Phosphatase	MBI Fermentas, St. Leon-Rot
DNAse I, RNAse free	Roche, Mannheim
Restriction Endonucleases	NEB, Frankfurt/ Main
	MBI Fermentas, St. Leon-Rot
T4-DNA Ligase	Roche, Mannheim
LA Taq-DNA Polymerase	Takara, Japan
Pfu DNA Polymerase	MBI Fermentas, St. Leon-Rot
Trypsin (GibCo)	Invitrogen, Eggenstein

1.3 „Kits" and other materials

Cell culture materials	Greiner, Solingen
	Nunclon, Dänemark
	Falcon, UK
Cellulose nitrate 0.45 µm	Schleicher & Schüll, Dassel
ECL Kit	PerkinElmer/NEN, Köln
Dual-Luciferase® Reporter Assay System	Promega, Mannheim
Glutathione-Sepharose	Amersham Pharmacia, Freiburg
Hyperfilm MP	Amersham Pharmacia, Freiburg
Micro BCA Protein Assay Kit	Pierce, Sankt Augustin
Parafilm	Dynatech, Denkendorf
Poly Prep® Chromatography columns	Bio-Rad, München
Protein A-Sepharose	Amersham Pharmacia, Freiburg
Protein G-Sepharose	Amersham Pharmacia, Freiburg
QIAquick Gel Extraction Kit (50)	Qiagen, Hilden

QIAquick PCR Purification Kit (50)	Qiagen, Hilden
QIAGEN Plasmid Mini Kit	Qiagen, Hilden
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden
Sephadex G-50 (DNA Quality)	Amersham Pharmacia, Freiburg
Sterile filter 0.22 µm, cellulose acetate	Nalge Company, USA
Sterile filter 0.45 µm, cellulose acetate	Nalge Company, USA
Whatman 3MM	Whatman, Rotenburg/Fulda

1.4. Growth factors and ligands

EGF (murine)	Toyoba, Japan
Insulin	Lilly, Giessen

2. Media

2.1. Bacterial media

LB or 2xYT media were used for cultivation of all *Escherichia coli* strains. If and as required 100 µg/ml Ampicillin or 70 µg/ml Kanamycin were added to media after autoclavation. For the preparation of LB-plates 1.5% Agar were also added.

LB-Medium	1.0	%	Tryptone
	0.5	%	Yeast Extract
	1.0	%	NaCl
			pH 7.2
2xYT-Medium	1.6	%	Tryptone
	1.0	%	Yeast Extract
	1.0	%	NaCl
			pH 7.2

2.2. Cell culture media

Gibco™ media, Ultroser G serum replacement and additives were obtained from Invitrogen (Eggenstein). Media were supplemented to the requirements of each cell line. Freeze medium contained 90% heat-inactivated FCS and 10% DMSO.

3. Stock solutions and commonly used buffers

BBS (2x)	50.0	mM	BES
	280.0	mM	NaCl
	1.5	mM	Na_2HPO_4
			pH 6.96
HEBS (2x)	46.0	mM	HEPES
	274.0	mM	NaCl
	1.5	mM	Na_2HPO_4
			pH 7.00
HNTG	20.0	mM	HEPES, pH 7.5
	150.	mM	NaCl
	0.1	%	TritonX-100
	10 .0	%	Glycerol
	10.0	mM	$Na_4P_2O_7$
DNA loading buffer (6x)	0.05	%	Bromphenol blue
	0.05	%	Xylencyanol
	30.0	%	Glycerol
	100.0	mM	EDTA pH 8.0
Laemmli buffer (2x)	65.0	mM	Tris/HCl pH 6.8
	2.0	%	SDS
	30.0	%	Glycerol
	0.01	%	Bromphenol blue
	5.0	%	ß-Mercaptoethanol
Laemmli buffer (3x)	100	mM	Tris/HCl pH 6.8
	3.0	%	SDS
	45.0	%	Glycerol
	0.01	%	Bromphenol blue
	7.5	%	ß-Mercaptoethanol
NET	50.0	mM	Tris/HCl pH 7.4
	5.0	mM	EDTA
	0.05	%	Triton X-100
	150.0	mM	NaCl
PBS	137.0	mM	NaCl
	27.0	mM	KCl
	80.9	mM	Na_2HPO_4
	1.5	mM	KH_2PO_4 pH 7.4
SD-Transblot	50.0	mM	Tris/HCl pH 7.5
	40.0	mM	Glycine
	20.0	%	Methanol
	0.004	%	SDS

"Strip" buffer	62.5	mM	Tris/HCl pH 6.8
	2.0	%	SDS
	100.0	mM	β-Mercaptoethanol
TAE	40.0	mM	Tris/Acetate pH 8.0
	1.0	mM	EDTA
TE10/0.1	10.0	mM	Tris/HCl pH 8.0
	0.1	mM	EDTA pH 8.0
Tris-Glycine-SDS	25.0	mM	Tris/HCl pH 7.5
	200.0	mM	Glycine
	0.1	%	SDS

4. Cells

4.1. Eukaryotic cell lines

Cell Line	Description	Origin/ Reference
786-0	Human primary renal cell carcinoma	ATCC, USA
ACHN	Human primary renal cell carcinoma	ATCC, USA
BT-20	Human mammary adenocarcinoma	ATCC, USA
Caki-1	Human renal metastatic cell carcinoma	ATCC, USA
COS-7	African green monkey, SV40-transformed kidney	Genentech, USA
HEK 293T	Human embryonic kidney fibroblasts, transformed with adenovirus Typ V DNA	ATCC, USA
HEK 293T-RPTPκEC	HEK 293T, transfected with pcDNA3-RPTPκEC-GST	ATCC, USA
HEK 293T-RPTPμEC	HEK 293T, transfected with pcDNA3-RPTPμEC-GST	ATCC, USA
Lovo	Human colon carcinoma	ATCC, USA
MDA-MB-453	Human mammary carcinoma	ATCC, USA
MDA-MB-468	Human mammary adenocarcinoma	ATCC, USA
Phoenix A	Packaging cell line for the generation of helper free amphotropic retroviruses, based on HEK 293T	G. Nolan, Stanford, USA
Phoenix E	Packaging cell line for the generation of helper free ecotropic retroviruses	G. Nolan, Stanford, USA

4.2. *E. Coli* strains

E. Coli strain	Genotype Description	Origin/ Reference
DH5αF'	F' endA1 hsd17 ($r_k^- m_k^+$) supE44 recA1 gyrA (Nal) thi-1 Δ(lacZYA-argF196)	Genentech, USA
XL1-Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F'proAB $lacI^q ZΔM15$ Tn10 (Tet^r)]	Stratagene, NL
BL21 Codon+	F' ompT hsdS(r_B– m_B–) dcm+ Tet^r gal λ (DE3) endA Hte (argU ileY leuW Cam^r)	Stratagene, NL
SCS110	rpsL (Str^r) thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44D (lac-proAB) (F' traD36 proAB $lacI^q ZΔM15$)	Stratagene, NL

5. Antibodies

Names of people given as reference without further designation were members of this group.

5.1. Primary antibodies

The following antibodies were used for immunoprecipitations or as primary antibodies in immunoblot and immunofluorescence analysis.

Antibody	Description/ Immunogen	Origin/ Reference
ß-Actin	Rabbit, polyclonal, directed against a C-terminal peptide	Sigma, Taufkirchen
β-Catenin	Mouse, monoclonal, binds C-terminal part of ß-Catenin	Transduction Laboratories, USA
β-Catenin	Rabbit, polyclonal, recognizes amino acids 373-781	Thomas Müller
EGFR	Mouse, monoclonal, recognizes amino acids 996-1022 of human EGFR	Transduction Laboratories, USA
EGFR	Sheep, polyclonal, binds to cytoplasmic part of human EGFR	UBI, USA
ERK2 (K-23)	Rabbit, polyclonal, peptide from sub-domain XI of rat ERK2, used for WB	Santa Cruz, USA
GST	Rabbit, polyclonal, binds to full length GST	Pjotr Knyazev
HA.11	Mouse, monoclonal, recognises the influenza hemagglutinin epitope	BAbCo, USA

HA (12CA5)	Mouse, monoclonal, recognises the influenza hemagglutinin epitope	Roche, Mannheim
JNK (C-17)	Rabbit, polyclonal, directed against a C-terminal peptide of human JNK1	Santa Cruz, USA
$p27^{Kip1}$	Mouse, monoclonal, recognizes full length p27 protein	Transduction Laboratories, USA
PCP-2-EC	Rabbit, polyclonal, binds extracellular fragment of PCP-2	This study
P-ERK2	Rabbit, polyclonal, recognises phospho-ERK1/2 (Thr-202/ Tyr-204) MAPK	NEB, Frankurt/M
P-JNK	Rabbit, polyclonal, recognises phospho-JNK (Thr183/Tyr185)	NEB, Frankfurt/M
Plasminogen	Mouse, monoclonal, binds to kringles 1-3 of human Plasminogen	Calbiochem, USA
Plasminogen	Rabbit, polyclonal, recognizes human Plasminogen	American Diagnostica, USA
Plasminogen	Gaot, polyclonal, recognizes human Plasminogen	Kordia, USA
P-Tyr (4G10)	Mouse, monoclonal, recognises phospho-tyrosine residues	UBI, USA
RPPTκ-EC	Rabbit, polyclonal, binds extracellular fragment of RPTPκ	This study
RPTPκ-JM	Rabbit, polyclonal, binds juxtramembrane fragment of RPTPκ	Marta Murgia
RPTPκ-JM	Goat, polyclonal, binds juxtramambrane fragment of RPTPκ	Marta Murgia
RPTPμ-EC	Rabbit, polyclonal, binds extracellular fragment of RPTPμ	This study
RPTPμ-MAM	Mouse, monoclonal, binds MAM-domain of RPTPμ	This study
α-Tubulin	Mouse, monoclonal, ascites	Sigma, Taufkirchen
VSV (P5D4)	Mouse, monoclonal; recognises an epitope of eleven amino acids derived from the vesicular stomatitis virus glycoprotein VSV-G	Roche, Mannheim

5.2. Secondary Antibodies

For immunoblot and immunofluorescence analysis corresponding secondary antibodies conjugated with horseradish peroxidase (HRP) or the fluorescent dye C2 were utilised.

Antibody	Dilution	Origin
Goat anti-mouse-HRP	1 : 20,000	Sigma, Taufkirchen
Sheep anti-goat-HRP	1 : 10,000	Sigma. Taufkirchen
Goat anti-rabbit-HRP	1 : 40,000	BioRad, München
Goat anti-rabbit-Cy2	1 : 1000	Jackson ImmunoResearch Labs, USA
Goat anti-mouse-Cy2	1 : 1000	Jackson ImmunoResearch Labs, USA

6. Plasmids and oligonucleotides

6.1. Primary vectors

Vector	Description	Origin/ Reference
pcDNA3	Mammalian expression vector, Amp^r, Neo^r, CMV promotor, BGH poly A, high copy number plasmid, F1+ origin	Invitrogen, USA
pcDNA3-Fc	Modified pcDNA3 containing cds for Fc chain 5' of the multiple cloning site	C.Cant
pcDNA3-GST	Modified pcDNA3 containing cds for GST chain 5' of the multiple cloning site	C.Cant
pBlueScript KS+	Cloning vector, F1 origin, Amp^r	Stratagene, USA
pGEX5X1-3	Prokaryotic expression vectors for the generation of glutathione-S-transferase fusion proteins, Amp^r, IPTG inducible	Amersham Pharmacia, Freiburg
pRK5	Expression vector, Amp^r, CMV promoter, SV40 poly A	Genentech

6.2. Constructs

Vector	Insert description	Reference
pEGFP-C2	cDNA of the enhanced green fluorescent protein (EGFP(	Clontech
pGL-3-CMV	cDNA of luciferase, CMV	B. Biesinger
pRK5-c-Src YF	cDNA of p60 c-Src constitutively active variant	(Luttrell et al., 1997)
pRK5-Fyn	cDNA of p59 Fyn	(Margolis et al., 1992)
pRK5-Dyn I	cDNA of Dyn I	Reiner Lammers
pRK5-Dyn I K44A	cDNA of Dyn I K44A Dom.-neg. mutant	Reiner Lammers
pRK5-RPTPκ	cDNA of RPTPκ	Miriam Fuchs
pRK5-RPTPκ-C/A1	cDNA of RPTPκ-C/A1 Catalytical C to A mutant PTP domain I	Miriam Fuchs
pRK5-RPTPκ-C/A1-HA	cDNA of RPTPκ-C/A1-HA Catalytical C to A mutant PTP domain I	Miriam Fuchs
pRK5-RPTPκ-C/S1	cDNA of RPTPκ-C/S1	This work
pRK5-RPTPκ-HA	cDNA of RPTPκ-HA	Miriam Fuchs
pRK5-RPTPκISi	cDNA of RPTPκISi S2 ckeavage site mutant	This work
pRK5-RPTPκLNTR	cDNA of RPTPκ LNTR Furin cleavage site mutant	Miriam Fuchs
pRK5-RPTPκLNTR-HA	cDNA of RPTPκ LNTR-HA Furin cleavage site mutant	Miriam Fuchs
pcDNA3-RPTPκMAM-GST	cDNA of RPTPκ MAM domain fused to GST	This work
pcDNA3-RPTPμMAM-GST	cDNA of RPTPμ MAM domain fused to GST	This work
pcDNA3-RPTPκEC-GST	cDNA of RPTPκ EC fragment fused to GST	This work
pcDNA3-RPTPμEC-GST	cDNA of RPTPμ EC fragment fused to GST	This work
pcDNA3-Furin	cDNA of Furin	Marta Murgia

6.3. Important Oligonucleotides

Sequence (description)	Name
5’ CCG GAA TTC GCC GCC ACC ATG GAT ACG ACT GCG GCG GCG 3’	RPTPκ MAM for
5’ CCG CTC GAG TGC ATT CAC CTC TAC ATC CCC 3’	RPTPκ MAM rev
(Cloning of the RPTPκ MAM GST fusion construct)	
5’ CCG GAA TTC GCC GCC ACC ATG AGG ACA CTT GGG ACT TGC 3’	RPTPμ MAM for
5’ CCG CTC GAG AGC ATT AAC TTC CAC ATT CTG 3’	RPTPμ MAM rev
(Cloning of the RPTPμ MAM GST fusion construct)	
5’ CAC TCT GTC TGT CGT CTT GGC GTC CTT GTC ATC GTC TTT GTA ATC TAC ACT GGG ATC TGG GAT CAC TTC TGG 3’	RPTPκ ISi rev
5’ CCA GAA GTG ATC CCA GAT CCC AGT GTA GAT TAC AAA 3’	RPTPκ ISi for
(Cloning of RPTPκ S2 cleavage inhibitory mutant)	
5’ CCC ATC GTT GTA CAT AGC AGT GCT GGT GCT GGA C 3’	RPTPκ C/A1 for
5’ GTC CAG CAC CAG CAC TGC TAT GTA CAA CGA TGG G 3’	RPTPκ C/A1 rev
5’ GCC CGC CAC CAT TTA GGC TGT GGA TAA TCG TCC GGC 3’	RPTPκ C/A2 for
5’ GGC CGG ACG ATT ATC CAC AGC CTA AAT GGT GGC GGG 3’	RPTPκ C/A2 rev
5’-GGAGCCGGAGCCATGGAATGC-3’	RPTPκ P-su for
5’-ATGGCTCCGGCTTCCGATCCCAGGA GAGGCCAAGGAGAGA GGAGCAAG-3’	RPTPκ P-su rev

7. Methods of Molecular Cloning

7.1. Plasmid Preparation

Small amounts of plasmid DNA were prepared using the Qiagen Plasmid Mini Kit, larger amounts of DNA were obtained with the Qiagen Plasmid Maxi Kit following the manufacturer’s instructions.

7.2. Enzymatic manipulation of DNA

7.2.1. Specific digestion of DNA samples by sestriction endonucleases

The ratio of Enzyme/DNA, the temperature, the buffer and the time of incubation were adjusted according to manufactures instruction. Usually, incubations for 2 hour at 37°C with a calculated 5-fold overdigestion and the buffers as supplied by the manufacturers were chosen.

7.2.2. Dephosphorylation of DNA 5'-termini

In order to prevent self-ligation of vector termini generated by restriction digest, 5´-termini of vector were dephosphorylated with Calf Intestine Alkaline Phosphatase (CIAP). This phosphatase removes 5´-phosphate residues from DNA as well as RNAFor dephosphorylation, 1 μg of cut vector DNA was incubated with 5 units CIAP in adequate reaction buffer (e.g. 50 mM Tris/HCl pH 8.0, 0.1 mM EDTA pH 8.5) at 37°C for 10 minutes. Either reactions were stopped by heat inactivation at 85°C for 10 minutes or DNA was directly purified using the QIAquick PCR Purification Kit.

7.2.3. Ligation of vector and insert DNA

Purified, digested and dephosphorylated vector DNA (40 ng), the designated insert DNA, 1 µl 10x T4 DNA Ligase buffer (0.66 M Tris/HCl pH 7,5, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP) and 1 unit T4 DNA Ligase were combined. A molar ratio between insert and vector of 3 to 1 was usually chosen. Reactions were either left on 14°C over night or at 37°C for 2 hours and subsequently transformed into competent bacteria.

7.2.4. Agarose gel electrophoresis

Depending on the size of the fragments of interest 0.7-2% agarose gels were prepared in horizontal chambers. TAE buffer was used for the electrophoresis. Voltage was usually set to 4-10 V per cm width of the gel. After separation DNA fragments were stained by gently agitating gels in TAE containing 0.5 µg/ml ethidium bromide and were subsequently viewed under UV light.

7.2.5. Isolation of DNA fragments from agarose gels

Following gel electrophoresis gel slices bearing DNA fragments of interest were cut out of the gel. Agarose was dissolved and DNA was purified using the QIAquick Gel Extraction Kit following Qiagen's protocol.

7.3. Introduction of plasmid DNA into *E.coli*

7.3.1. Preparation of competent cells

The preparation of competent cells was according to the procedure described by Chung and Miller (Chung and Miller, 1993). Competent cells were shock frozen in liquid nitrogen and stored for up to one year at –70°C. Transformation frequency ranged between 10^5 and 10^7 colonies/µg DNA.

7.3.2. Transformation of competent bacteria

A 50 µl aliquot of competent bacteria was added to a 50 µl mixture of DNA usually ligation cocktails, 10 µl 5x KCM solution (500 mM KCl, 150 mM $CaCl_2$, 250 mM $MgCl_2$) and water. After thoroughly mixing, samples were incubated on ice for 20 minutes, 10 minutes at room temperature and after addition of 300 µl LB broth at 37°C for 1 hour while constantly shaking. Bacteria were streaked out on appropriate agar plates containing Ampicillin for the selection of transformants.

7.4. Site-directed mutagenesis using double-stranded template DNA

Stratagene's QuikChange method was used for site-directed mutagenesis using double-stranded template DNA, e.g. introduction of point muatations or deletions. Pfu-based PCR reactions, Dpn I digests and transformations were performed according to Stratagen's instructions.

7.5. Enzymatic amplification of DNA by polymerase chain reaction (PCR)

For long and accurate cDNA amplification Pfu Polymerase (MBI Fermentas) was used:

1 µl	template cDNA, 1-10 ng
1 µl	"forward" oligonucleotide, 10 pmol/µl
1 µl	"reverse" oligonucleotide, 10 pmol/µl

2.5 µl	10x PCR buffer II containing 20 mM $MgCl_2$
2 µl	dNTP-Mix, 2.5 mM each
0.5 µl	Pfu DNA Polymerase (2.5 U/µl)
ad 25 µl	H_2O

PCR reactions were carried out using an automated thermal cycler („Progene“, Techne). The following standard protocol was adjusted to each specific application:

3 min 94°C	(initial denaturation)
30 cycles:	
1 min 94°C	(denaturation)
1 min 58°C	(hybridization)
1.5 min/ kb	
5 min 72°C	(extension)

PCR products were either separated by agarose gel electrophoresis, excised and subsequently purified or directly purified with QIAquick Gel Extraction or PCR Purification Kit, respectively.

7.6. DNA sequencing

Sequencing of DNA was performed following the “Big Dye Terminator Cycle Sequencing Protocol” (ABI). Pellets were dissolved in 20 µl template suppression reagent, briefly boiled and analysed on a 310-Genetic Analyzer (ABI Prism).

7.7. Isolation and fractionation of RNA and cDNA synthesis

Total RNA was isolated from the cell pellet by lysis in guanidinium isothiocyanate solution (GTS buffer: 4 M guanidinium isothiocyanate, 25 mM sodium citrate pH 7.0, 0.5% Sarkosyl, and 0.1 M ß-mercaptoethanol) followed by phenol-chloroform extractions (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=2440339). mRNA extraction was performed using the OligoTex mRNA isolation kit (Quiagen, Biotech, Germany) or with Oligo-dT cellulose (Stratagene Inc.). The Oligo-dT cellulose column was washed three times with lysis/binding buffer and four times with washing buffer before eluting the mRNA with pre-warmed (65°C) elution buffer. Total and mRNA integrity and cDNA complexity was controlled by agarose gel electrophoresis and northern blots using specific probes. The quantity of mRNA was measured by optical density (OD260). cDNAs were synthesized on 3µg of mRNA in presence of oligo-dT and random primers using 100 units of MoMLV reverse-transcriptase, RNaseH-free (Promega Inc, USA).

7.8. RNA analysis by Northern-blot

For analysis of the RPTPκ messenger in preparations derived from kidney tumors and surrounding normal tissue a northern blot standard protocol was used according to Sambrock, Fritsch and Maniatis (1989). Loading of RNA samples were verified by rehybridization of filters with a human β-actin probe.

8. Cell culture and transfections

All of the cell lines (American Type Culture Collection, Manassas, VA) were routinely grown according to the supplier's instructions. To analyse cell density-dependent cleavage, MDA-MB-453 cells and MDA-MB-468 cells were incubated in the serum substitute Ultroser G as indicated. HEK293 and LoVo cells were transfected by using a modified calcium phosphate-DNA co-precipitation method as described (Jordan et al., 1996). Transiently transfected HEK293 cells were assayed and lysed 24h after removal of precipitates. Monoclonal LoVo cell clones stably expressing furin were obtained after selection in the presence of G418 (500µg/ml). COS7 cells were transiently transfected with Polyfect (Qiagen) according to the manufacture's instructions. Tranfection effciency was determined using reporter plasmids, such as CMV promotor driven GFP and Luciferase expression constructs. While GFP expression was easily monitored by fluorescence microscopy, relative luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega).

9. Protein analytical methods

9.1. Cell treatment and extract preparation

In case of using RTK ligands, cells were seeded in cell culture dishes of appropriate size and grown over night to 30% confluence. After serum withdrawal, again over night, cells were stimulated with EGF (200ng/ml) as indicated in the figure legends. Stimulations were stopped by washing cells with PBS and immediate lysis. In case of using shedding inducers or protease inhibitors, cells were grown to confluence and the compounds were added as indicated. All compounds were kept as DMSO stock solutions. The furin inhibitor CMK was included in the incubation mixture at 100 µmol/L. If not otherwise indicated, all cells were treated with phenothiazine derivatives trifluoperazine, chlorpromazine, promazine and fluophenazine at a concetration of 100µM. The phorbolester PMA and ionomycin were used at a concentration of 1µM. Proteosomal and lysosomal protein degradation was inhibited with 10µM MG132. To this end, confluent 786-O cells were treated with MG132 for 4 and 8h. In order to inhibit Zn-dependent proteases, cell were treated with BB-94 (batimastat) at a concentration of 5µM. Following 20min of BB-94 incubation, TFP was added to cells for 15min. Methyl-ß-cyclodextrin (MßCD) was added to 786-O cells at a concentration of 10mM for 20min prior to TFP stimulation for 15min. Cells were lysed and proteins solubilized on ice for 30min in ice-cold lysis buffer (50mM HEPES, pH7,5; 150mM NaCl; 2mM EDTA; 10% glycerine, 1% Triton X-100; 10mM $Na_4P_2O_7$; 1mM PMSF, 0,1µg/ml aprotinin, 10mM NaF, 1mM Na_3VO_4).

9.2. Preparation of crude cell lysates from tissue

The tissue material was powderized under liquid nitrogene conditions, followed by solubilization in ico-cold lysis buffer as described above. To allow appropriate solubilization, samples were incubated for 2h on a shaker at 4°C. Following centrifugation, supernatants were filtered to separate fat particles from the protein extraction and the protein concentration was adjusted to 3mg/ml. Immunoprecipitations and preparation of total lysates were performed as decribed elsewhere.

9.3. Determination of total protein concentration in lysates

The overall protein concentration was determined using the Micro BCA Protein Assay Kit (Pierce, Sankt Augustin) and the supplied standard protocol.

9.4. Immunoprecipitation and western blotting

Endogenously expressed RPTRκ was immunoprecipitated with antibody αRPTPκJM. After SDS-PAGE, proteins were transferred to nitrocellulose membrane and Western blots were performed according to standard protocols. HA-tagged RPTPκ was detected from whole cell lysate by using antibody αHA. The P-subunit was detected with antibody αRPTPκJM, the E-subunit and the extracellular P-stulk with antibody αRPTPκEC. Cell supernatants were analysed for accumulation of κ-specific fragments by TCA precipitation and detection of fragments by using antibody αRPTPκEC.

9.5. TCA protein precipitation

TCA precipitation was used to concentrate proteins from cell supernatants for analysis by SDS-PAGE. Cells were washed twice with serum-free medium. Following cell stimulation or basal shedding, cell media were centrifuged at 2000g for 5min to pellet detached cells. Afterwards, supernatants we centrifuged at 20000g to remove cell debris and subjected to TCA precipitation. Briefly, samples were mixed with an equal volume of 20% TCA, incubated on ice for 30min, centrifuged for 15min and supernatants carefully removed. Pellets were washed in ice-cold acetone and centrifuged for 10min, dried, resuspended in SDS-PAGE-loading buffer and heated at 65°C for 3min.

9.6. In vitro Plasminogen-binding studies

Cell media containing EC-GST or MAM-GST fusion proteins were processed by GST affinity chromatography by using the Äkta Explorer System (Amersham Biotech) in connection with a GST HiTrap 1ml column. Bound proteins were eluted according to the manufacture's protocol. Elutions were mixed in 3x Laemmli's buffer, boiled and processed to SDS-PAGE. Coeluted proteins were identified by an approach using proteolytic digestion and internal EDMAN sequencing. For in vitro binding studies, 5mg of either BSA or Plasminogen were immobilized to CNBr sepharose beads (Amersham Biosciences) according to the manufacturer's instructions and were incubated with 10µg of EC-GST or MAM-GST fusion protein in 600 µl PBS (8 mM Na_2HPO_4, 1,5 mM KH_2PO_4, 137 mM NaCl, 2,7 mM KCl, pH 7,3) for 16h in an end-over-end rotator. Afterwards, beads were washed four times with 1ml HNTG buffer and heated in SDS-PAGE-loading buffer at 100°C for 3min.

9.7. Quantification of immunoblot signals

For quantification of protein bands obtained from Western Blots we used the Pharmacia LKB ImageMaster DTS. Signals were analysed by laser scanning using the program *Quantity One*^{R} from pdi (Oakwood, New York). Obtained signal peak intensities were integrated and the values used for further calculations as decribed in the respective figure legends.

9.8. Screening of cellulose-bound peptides libraries

Peptide libraries were prepared by automated spot synthesis (Frank, 1992; Kramer et al., 1994; Kramer und Schneider-Mergener, 1998), in which peptides are C-terminally attached to cellulose via (ß-Ala)$_2$ spacer. Peptides were derived from the MAM domain of RPTPκ. The screening followed a published procedure (Rüdiger et al., 1997) with slight modifications. Before screening the dry membranes were washed for 2 times 10 min in Methanol and 5 times

15 min in TBS, pH 7,6. Plasminogen (0,02%) was allowed to react with peptide scans in TBS/0,05% Tween 20 o.N. at 4 deg with gentle shaking. Unbound Plasminogen was removed with TBS/0,05% Tween and peptide-bound Plasminogen was electrotransferred on to polyvinylene difluoride (PVDF) membranes (Millipore) using a semidry blotter. Transferred Plasminogen was detected using Plasminogen-specific antibody as described under 9.9.

9.9. In vitro phosphatase assay

We used *para*-Nitrophenylphosphate (pNPP) assays for quantification of in vitro phosphatase activity. MDA-MB-468 cells were lysed in the absence of phosphatase inhibitors and proteins immunoprecipitated using antibody αRPTPκJM or preserum as control. Precipitates were washed and incubated with reaction buffer containing 100mM 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 5.5, 10mM dithiothreitol, 150mM NaCl, 2mM EDTA and 100mM *para*-nitrophenylphosphate at 37° in an end-over-end rotator. 1.0M NaOH was added to samples at different time points and $A_{415\,nm}$ values were measured spectrophotometrically. The linear slope of the absorbance-time curve obtained from the initial course of the reaction was calculated.

10. Protein expression and purification

10.1. Expression of GST fusion proteins in HEK 293 cells

We performed large scale (1-10 L) transient expression from HEK293 cells for rapid production of GST-fusion proteins. Adherent HEK293 cells were expanded in DMEM supplemented with 10% FCS and splitted in Nunclon delta SI cell culture boxes. Transfections were performed according to the Calcium Phosphate Precipitation method described above. 12 h posttransfection, the medium was changed to DMEM containing 1% FCS and proteins were synthesized within a timeframe of 7 days from time of transfection to harvest. All proteins were secreted into the supernatant of the cell culture.

10.2. Purification of GST fusion proteins

Cell supernatants were collected, centrifuged and filtered through a 0,22µm syringe. For purification we used the Äkta Explorer System (Amersham Biotech) in connection with a GST HiTrap 1ml column. The column was washed with PBS and proteins eluted with 50mM Tris-HCl, 10mM glutathion, 1mM DTT and 1mM EDTA, pH 8,0. Purified proteins were dialysed against 10% Glycerol in PBS. GST-fusion proteins were used for (1) immunization of mice, (2) immunization of rabbits and (3) biochemical and cell biological assays (see below).

11. Biochemical and cell biological methods

11.1. Membrane fractionation

Cells were washed and scraped in PBS, pelleted by centrifugation (5min, 12000rpm) and incubated in hypotonic buffer (20mM HEPES, pH 7,2; 10mM KCl; 1,5mM $MgCl_2$; 0,1mM EGTA; 10mM EDTA; 10mM $Na_4P_2O_7$; 1mM PMSF, 0,1µg/ml aprotinin, 10mM NaF, 1mM Na_3VO_4) for 30min on ice. Cells were broken using a dounce homogenizer (30 strokes) and nuclei were pelleted by centrifugation (10min, 3500rpm). Nuclei-free supernatant was subjected to ultracentrifugation at 100000g for 1h to separate the membrane (pellet) from the

cytosolic fraction (supernatant). The pellet was washed with hypotonic buffer and solubilized with membrane solubilization buffer, followed by centrifugation at 100000g for 1h. The resulting supernatant was termed membrane fraction.

11.2. Pulse chase labelling

Cells were seeded in 10cm dishes at approximately 50% confluence one day before the experiment was performed. 500µl fresh stock solution of (S^{35}) Met (5mCi/0,5m) was added to 50ml Met-free MEM in a Falcon tube (radioactive Premix) and warmed up at 37 deg. The experiment was performed in three steps: (I) Methionine starving: Cells were washed quickly two times with PBS and incubated in Methionine- and FCS- free MEM medium for 30min. (II) Pulse: 5ml radioactive Premix was added to each dish and cells incubated for 10-20 min (10-20 min-Pulse). (III) Chase: Cells were washed two times quickly with PBS and 10ml of 1000fold Methionine-excess containing medium was immediately added. Incubations were stopped at given time intervals and cells either frozen or lysed.

11.3. Potassium depletion

Cells were washed two times with Kalium depletion buffer (50mM HEPES, pH 7,4; 100mM NaCl; 1mM $CaCl_2$, 1mM $MgCl_2$), followed by incubation in DMEM/H_2O (1:1) for 5min. Cells were again washed two times with Kalium depletion buffer and incubated either in depletion buffer (Kalium depletion) or in appropriate medium without FCS (normal medium) at 37°C for 2h. Afterwards, 786-O were treated with 100µM TFP for 15min that was added to either depletion buffer and to regular medium. In order to inhibit basal shedding, ACHN cells were incubated for further 0,5 and 2h in depletion buffer.

11.4. Biotinylation of cell surface proteins

Cell surface proteins were biotinylated using the water soluble biotinylation reagent, ss-biotin, following a previously published procedure (Volz et al., 1995) with minor modifications. Briefly, cells were rapidly cooled on ice for 5 min and then washed with PBS at 4 °C. The cells were then incubated with freshly prepared sulfo-NHS-ss-biotin/PBS solution (1.0 mg/ml) at 4 °C for 45 min. After washing twice with PBS at 4 °C, the cells were immediately lysed and RPTPκ immunoprecipitated.

11.5. Analysis of cell density-dependent cleavage

Cells were plated and incubated at increasing cell densities for 24h in RPMI medium supplemented with Ultroser G serum substitute. For instance, MDA-MB-468 cells were seeded in P15 (low density), P10 (medium density) or P6 (high density) dishes as follows: P15: $3{,}4\times10^6$ cells, P10: $6{,}8\times10^6$ cells, P6: $13{,}6\times10^6$ cells. MDA-MB-453 cells were seeded as follows: P15: $4{,}3\times10^6$ cells, P10: $8{,}6\times10^6$ cells, P6: $17{,}2\times10^6$ cells.

IV. Results

MAM-family RPTPs function as homophilic cell-to-cell adhesion receptors and are localized at sites of cell contact (Brady-Kalney et al., 1993; Sap et al., 1994). RPTPκ was actually shown to bind ß-Catenin, a component of *adherent junctions* (Fuchs et al., 1996). Moreover, cells at conflunce increase the expression of κ (Fuchs et al., 1996). The κ protein is composed out of two non-covalently bound proteolytic fragments, called E- and P-subunit (Jiang et al., 1993).

The data presented in this thesis may be devided in three parts. The main one represents an investigation into the regulation of this phosphatase by proteolytic processing. We describe three differentially regulated cleavage events that all target different sites within the κ sequence. Evidence of deregulated processing of κ in human tumors is reported. Part two identifies a κ-specific antibody to the extracellular portion as "pseudeligand". Part three deals with the identification of potential RPTPκ "heterophilic" ligands.

1. Proteolytic processing of RPTPκ

1.1. S1 processing of RPTPκ

1.1.1. Furin is required for S1 processing of RPTPκ

The RPTPκ protein consists of two subunits: the transmembrane P-subunit (100kD), harbouring two PTP domains, and the extracellular E-subunit (120kD), covering most of the extracellular sequence. Both subunits are non-covalently attached to each other and are generated from one precursor protein by proteolyic processing at the dibasic cleavage site (RTKR) which is located within the membrane proximal fibronectin-type III domain (Jiang et al., 1993, Fuchs et al., 1996). Surface biotinlylation of HEK 293 cells transfected with wild type RPTPκ revealed that κ proteins presented at the cell surface are exclusively composed out of two subunits (Fig. 7A). In contrast, a cleavage-site mutant in which the RTKR motif was changed to LNTR is transported to the cell surface in the full-length form. So cleavage at RTKR seems to be part of a maturation process that proceeds within the secretory pathway.

Mammalian subtilisin-like protein convertases (PCs) mediate constitutive processing of precursor proteins in the *trans*-Golgi network. Four of them, namely furin, PACE4, PC5/6

and LPC, are ubiquitiously expressed and PC5 has been shown to mediate cleavage of RPTPμ (Campan et al., 1996), a closely related phosphatase of the MAM-family.

Analysis of RPTPκ protein expression in numerous cell lines showed that furin-deficient LoVo cells accumulate the precursor (mock-transfected LoVo cells, Fig. 7B). Importantly, LoVo cells do not express functional furin due to mutations in both Fur alleles (Takahashi et al., 1993; Takahashi et al. 1995) and reintroduction of furin into these cells by stable transfection restores κ cleavage (Fig. 7B) as did incubation of LoVo cell-derived RPTPκ with recombinant furin *in vitro* (Fig. 7C). Also, furin-mediated in vitro cleavage specifically targets the RTKR site since no additional fragments despite the E-and P-subunit could be observed. As a control, the furin inhibitor cmk (Garten et al., 1994; Seidah et al., 1994; Schäcke et al., 1998) was added to inhibit in vitro κ processing.

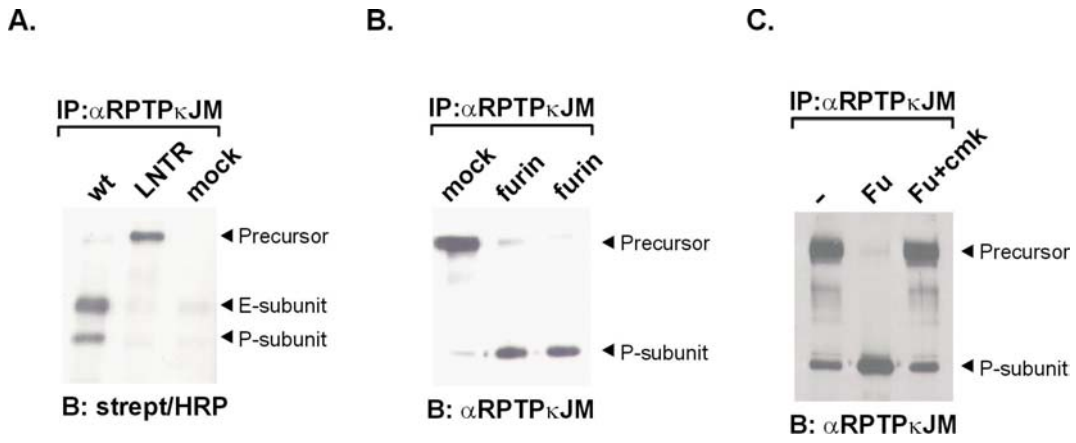

Figure 7: RPTPκ is a two-subunit receptor at the cell surface after Furin-mediated processing. *(A) Cell surface-presented RPTPκ is a two-subunit enzyme, composed of the extracellular (E) subunit and the PTP domain-containing (P) subunit. 293 cells were transfected with wt-RPTPκ and the convertase cleavage site mutant RPTPκ-LNTR, in which the dibasic sequence motif RTKR located in the membrane proximal fibronectin-type III domain was replaced by LNTR. Cells were surface-biotinylated prior to lysis under standard conditions as described under "Experimental procedures". RPTPκ was analysed by immunoprecipitation and western blotting using an antibody to the intracellular juxtamembrane part (αRPTPκJM). (B) Accumulation of the κ precursor in LoVo cells that are devoid of functional furin. The RPTPκ protein was analysed in a panel of colon carcinoma cell lines by immunoprecipitation with antibody αRPTPκJM.(C) Stable reexpression of furin in LoVo cells restores processing of κ. The phosphatase was immunoprecipitated from two LoVo cell clones stably expressing human furin and from a vector-transfected clone for comparison. (D) Purified furin cleaves κ at the sequence RTKR within the membrane-proximal fibronectin-type III domain in vitro. Furin-null LoVo cell-derived RPTPκ was immunoprecipitated and incubated for 1 h at 37°C with PBS (-), purified recombinant mouse furin (Fu) or purified recombinant furin previously treated with the inhibitor decRVKR-cmk (Fu + cmk).*

These results show that RPTPκ is a two-subunit receptor at the cell surface as a result of furin-mediated processing. In analogy to processing of the Notch protein by furin at the dibasic motif RQRR (Logeat et al., 1998), κ cleavage at RTKR is herein after referred to as S1 processing and RTKR as S1 site.

1.2. S2 processing of RPTPκ

1.2.1. Accumulation of RPTPκ P2 at high cell density

RPTPκ expression in several cell lines depends on cell density and highest expression is observed at cell confluence (Fuchs et al., 1996). Initially, we observed a previously undescribed κ-specific product of 75kD (hereafter designated P2 to specifiy the result of potential processing at site 2, a site downstream of S1; see below) which accumulated in high density cultures in a panel of breast, renal, melanoma and colon carcinoma cell lines. For instance, when MDA-MB-468 and MDA-MB-453 breast carcinoma cells were seeded at sparse (1), medium (2) and high density (3) and incubated for 24h, accumulation of P2 was found to be increased in high density as compared to low density cultures (Fig. 8A). In another approach, cells were seeded at low densities and incubated for different time intervals, i.e. one, two and three days, and so cells which have grown for longer time reached higher densities and showed increased amounts of P2 as well (Fig. 8B).

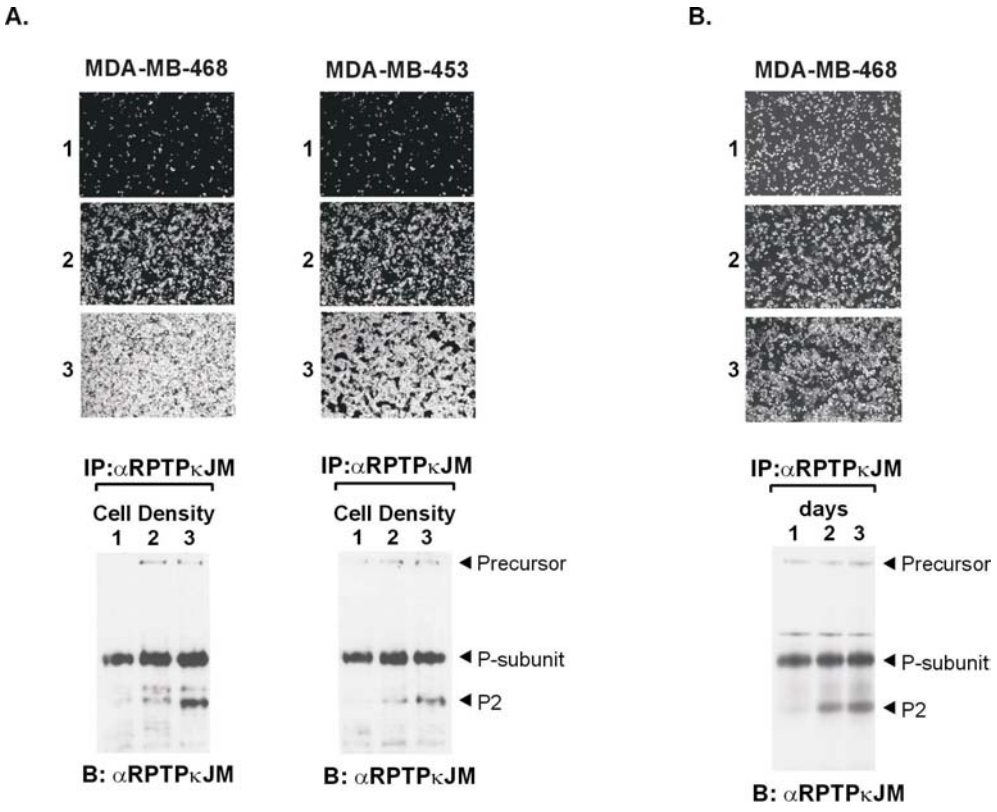

Figure 8: Accumulation of a P-subunit-derived κ product (P2) at high cell density. *(A). Mammary carcinoma cell lines MDA-MB-468 and MDA-MB-453 were seeded at increasing cell densities (10%, 50%, 100%) and incubated for 24h in serum-free medium containing 2% (v/v) Ultroser G. RPTPκ was immunoprecipitated with antibody αRPTPκJM and analysed as indicated. (B) MDA-MB-468 cells were seeded at equal cell density in medium containing 10% FCS and incubated for 1, 2 or 3 days.*

1.2.2. P2 accumulation and shedding of RPTPκ as a result of proteolytic processing at a second site (S2)

Since P2 accumulation in high cell density cultures is characterized by only slow induction, we sought to rapidly induce the product in a short-term assay. We hypothesized that generation of P2 is achieved by cleavage at an extracellular site close to the membrane that we named S2 (located downstream of S1). For instance, S2 cleavage of Notch has been demonstrated to result in shedding of its extracellular portion (Mumm et al., 2000). Moreover, lar family RPTPs were reported to be shed (Aicher et al., 1997). Initially, we have tested several compounds that are known as "common shedding inducers" like the phorbolester PMA, the Ca^{2+}-ionophore ionomycin (Io) and the phenothiazine derivative trifluoperazine (TFP) for their ability to induce κ cleavage at S2. For instance, TFP treatment of renal and mammary carcinoma cell lines resulted in S2 cleavage of κ. The extent of cleavage induced by ionomycin was only moderate, whereas PMA did not induce P2 accumulation (Fig. 9).

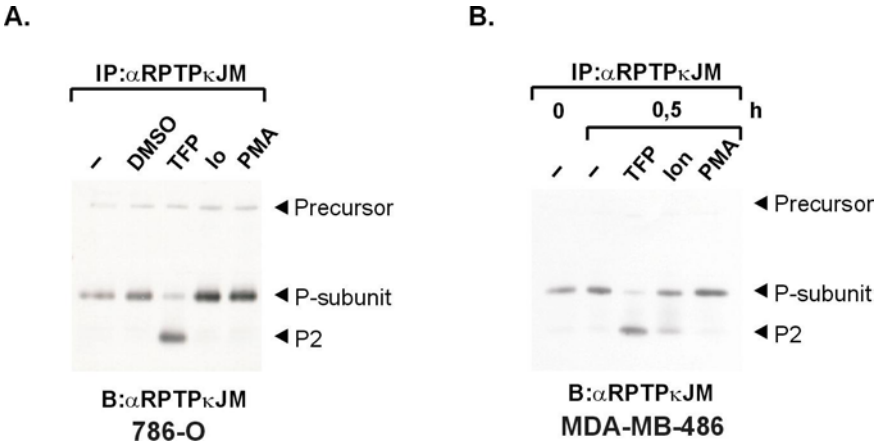

Figure 9: Proteolytic processing at a second site (S2 cleavage) results in generation of P2. *Trifluoperazine (TFP) induces cleavage of RPTPκ on site 2. 786-O renal carcinoma cells (A) and MDA-MB-468 mammary carcinoma cells (B) were treated with 1μM of the phorbolester PMA, 1μM of ionomycin (Io) and 100μM of the phenothiazine derivative trifluoperazine (TFP) for 30 min. RPTPκ was immunoprecipitated from the cell lysate and detected by immunoblotting using αRPTPκJM antibody to the intracellular juxtamembrane part.*

TFP is one derivative of phenothiazines, a class of drugs that are widely used to control mental disorders like schizophrenia, depressions and related conditions. To address the question of whether S2 processing is induced by phenothiazines in general, we tested several phenothiazine derivatives for their ability to induce κ cleavage. Fig. 10A shows that fluphenazine (FPZ) and trifluoperazine (TFP) were most effective in inducing cleavage at S2. Chlorpromazine (CPZ) treatment also provoked cleavage, whereas promazine (PMZ) was almost ineffective. These differences could be explained by the presence of piperazine-

substituted side chains in TFP and FPZ molecules and/or the addition of -Cl or -CF_3 residues to the phenothiazine ring structure (Fig. 10B).

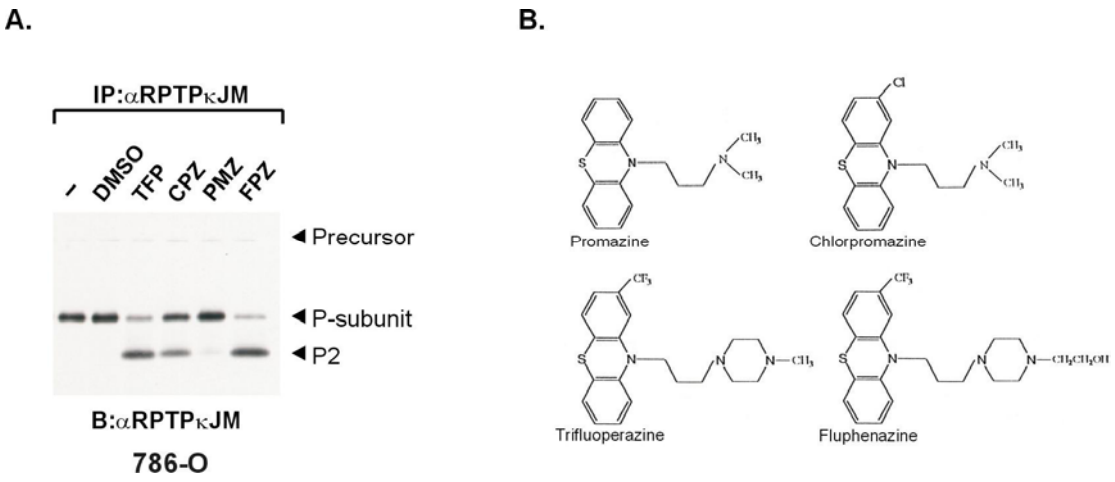

Figure 10: Phenothiazines induce S2 processing. *(A) 786-O renal carcinoma cells were treated with vehicle (DMSO) or 100µM of Trifluoperazine (TFP), Chlorpromazine (CPZ), Promazine (PMZ) or Fluphenazine (FPZ) for 20min. RPTPκ was immunopecipitated and analysed as indicated. (B) Chemical structures of TFP, CPZ, PMZ and FPZ.*

TFP-induced S2 processing was found to be time- and dose-dependend. Moreover, cleavage at S2 provoked the release of κ's extracellular portion from cells (Fig. 11A and B, middle panel), concomitant with the accumulation of the extracellular subunit in cell supernatants (Fig. 11A and B, lower panel). In fact more than 50% of the amount of the RPTPκ holo-receptor was cleaved and shed after 5min by treatment with TFP at a concentration of 100µM (Fig. 11B). The TFP effect on κ shedding was maximal at 75 to 100 µM TFP, and half maximal at TFP concentrations between 25 and 50µM.

To demonstrate that P2 is bound to the plasma membrane, we performed membrane fractionation experiments. As shown in Fig. 12, P2 was exclusively found in the membrane fraction and no κ-specific signals were detected in the cytosolic fraction (Fig. 12, upper panel). Taken together, S2 processing induced by phenothazines targets the ectodomain of RPTPκ, thereby leading to the generation of transmembrane P2 and shedding of the extracellular fragment.

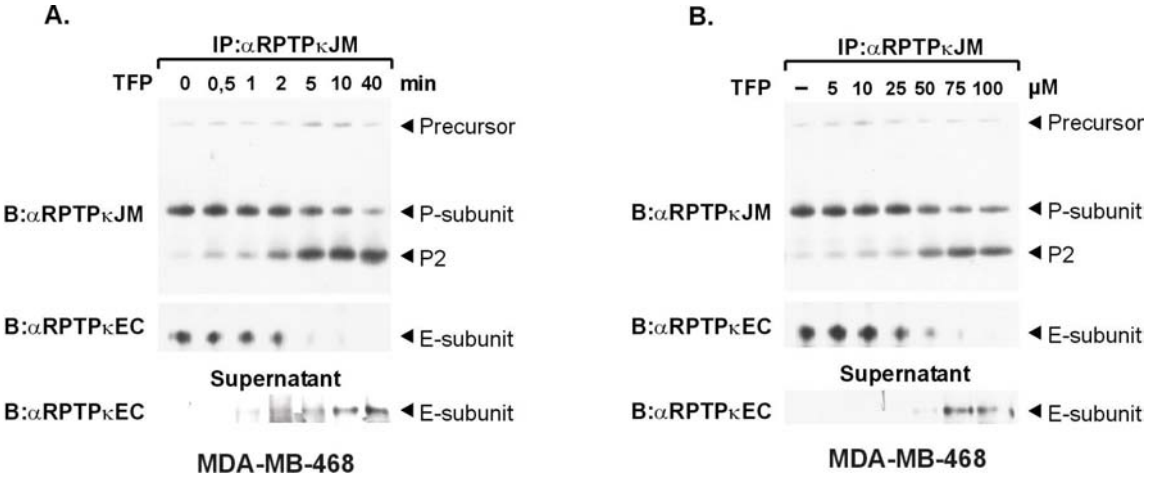

Figure 11: TFP-induced S2 cleavage results in shedding. *(A) Time-dependence of TFP-induced S2 cleavage and shedding. MDA-MB-468 cells were seeded at confluence, washed and incubated in serum-free medium. The cells were treated with 100µM TFP for different incubation times as indicated. Upper panel: RPTPκ was immunoprecipitated from cell lysate with antibody αRPTPκJM, blotted and probed with the same antibody. Middle panel: The blot was reprobed with antibody αRPTPκEC. Lower panel: Conditioned media were collected, proteins concentrated by TCA-precipitation, blotted and probed with an antibody to the extracellular part of RPTPκ (αRPTPκEC). (B) Concentration-dependence of TFP-induced S2 cleavage and shedding. MDA-MB-468 cells were incubated with varying concentrations of TFP for 20min as indicated. Cell lysates and supernatants were processed as described in A.*

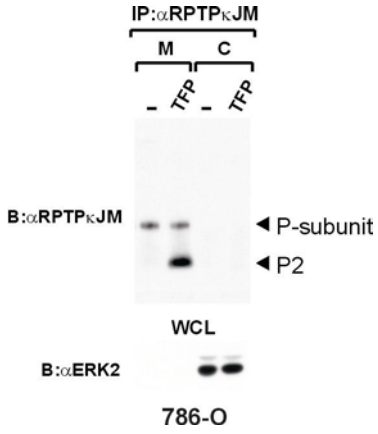

Figure 12: The P2 fragment is bound to the plasma membrane. *786-O cells were incubated with 100µM TFP for 30 min and resuspended in hypotonic buffer. Soluble, cytosolic (C) and membrane-bound proteins (M) were separated by membrane fractionation. Upper panel: RPTPκ was immunoprecipitated from both fractions and analysed by immunoblot as indicated. Lower panel: As a control, f ractions were analysed for the cytosolic marker protein ERK2 by immunoblotting.*

1.2.3. Metalloproteases account for S2 activity

It has been reported that Furin is involved in shedding of membrane-type matrix metalloproteinase 5 (Wang et al., 2001). In contrast, furin-mediated processing of RPTPκ yields two-subunit proteins (Fig. 13A) but we detected the E-subunit of κ upon TFP-induced cleavage in cell supernatants. To rule out the possibility that shedding is the result of κ subunit dissociation, we transfected κ and the furin cleavage-site mutant κ-LNTR into COS-7

cells and analysed the cell supernatants for κ-specific products. We hypothesized that, as a result of cleavage of the P-subunit close to the membrane (site 2), the short P-subunit-derived stulk accumulates in cell media in addition to the E-subunit. However, S2 processing of the furin cleavage-resistant precursor should not produce the P-stulk. Indeed, both TFP-induced and basal processing of wt-κ led to accumulation of the E-subunit plus the extracellular stulk of the P-subunit (Fig. 13A, B,lower panel) and cleavage of the LNTR-mutant did not yield the P-stulk, but instead led to the accumulation of a fragment larger then the E-subunit. We conclude that S2, but not S1 processing, results in shedding of RPTPκ.

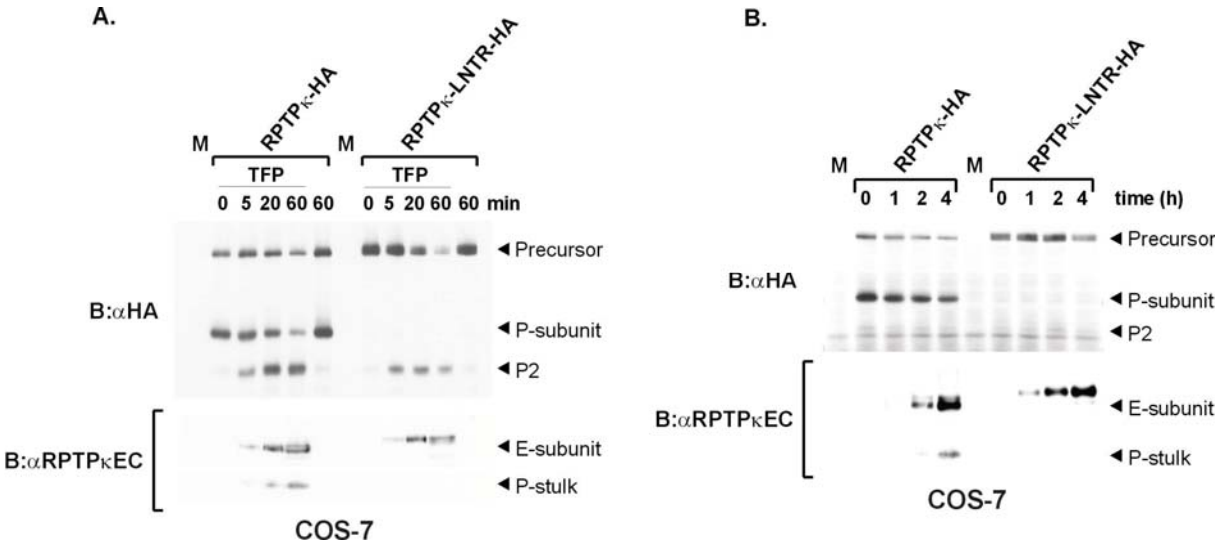

Figure 13: TFP-induced RPTPκ shedding and shedding at high cell density proceed via similar mechanisms, both of which are not due to subunit dissociation. *(A) The RPTPκ constructs used here are HA-tagged at the C-terminus. Time course of TFP-induced shedding of RPTPκ and RPTPκ-LNTR. COS-7 cells were transfected with cDNAs of RPTPκ, RPTPκ-LNTR or pRK5 vector (M). Cells were washed, incubated in serum-free medium and stimulated with TFP for the times indicated. Upper panel: Cell lysates were blotted and probed with antibody αHA. Lower panel: Detection of shed κ-specific fragments. Conditioned media were collected, proteins concentrated by TCA-precipitation, blotted and probed with antibody αRPTPκEC. (B) Time course of RPTPκ basal shedding at high cell density. Transfected COS-7 cells were incubated in serum-free medium for the times indicated. Cell lysates and conditioned media were processed as described in A.*

The identification of cell density as one physiological stimulus leading to S2 processing is interesting in regard to the homophilic binding behaviour of MAM-family RPTPs which is mediated via their E-subunits. Initially, to address the question if homophilic binding between κ proteins expressed in *trans* triggers their cleavage at S2, we generated a construct that is devoid of the E-subunit, i.e. we fused the P-subunit sequence directly to the putative κ signal peptide. We next transfected COS7 cells with wt-κ or the P-subunit mutant and analysed shedding upon basal and TFP-induced cleavage. Interestingly, in both cases we did not detect the P-stulk in cell supernatants in the absence of the E-subunit, indicating that the E-subunit is

required for RPTPκ shedding into the cell medium. (Fig. 14). However, analysis of the cell lysate revealed that the P-subunit is still cleaved when the E-subunit is missing. The discrepancy of these results could be explained, for instance, by compartmentalisation of the P-subunit into endocytic vesicles in such a way that shed P-stulk becomes internalised together with the S2 fragment. Altogether, these results demonstrate that TFP and cell density-induced shedding cause S2 cleavage via similar mechanisms, both of which lead to shedding of the P-stulk into the cell medium that requires, in both cases, the presence of the E-subunit.

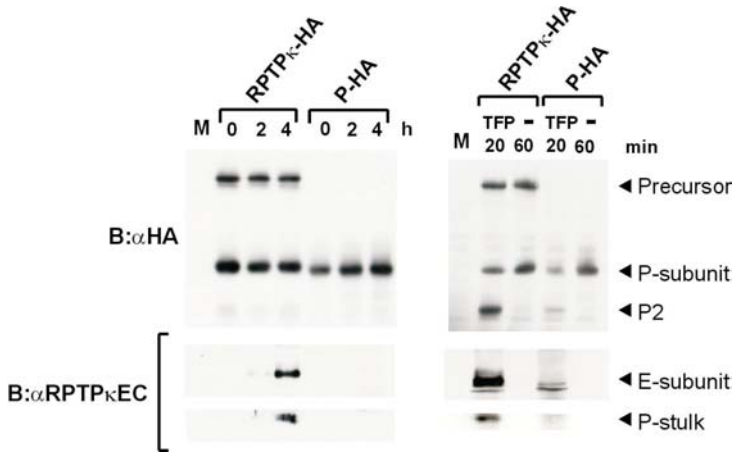

Figure 14: Both, TFP and cell density-induced shedding of the P-stulk require the presence of the E-subunit. *The RPTPκ constructs used here are HA-tagged at the C-terminus. Time course of TFP-induced shedding (left) and basal shedding (right) of RPTPκ and the RPTPκ P-subunit fused to the signal sequence. COS-7 cells were transfected with cDNAs of RPTPκ, the isolated P-subunit or pRK5 vector (M). Cells were washed, incubated in serum-free medium and stimulated with TFP for the times indicated. Upper panel: Cell lysates were blotted and probed with antibody αHA. Lower panel: Detection of shed κ-specific fragments. Conditioned media were collected, proteins concentrated by TCA-precipitation, blotted and probed with antibody αRPTPκEC.*

Membrane-integrated Zn-dependent proteases of the ADAM family have been implicated in shedding of numerous cell surface proteins (Seals and Courtneidge, 2003). In order to identify the enzymatic activity responsible for processing of RPTPκ at the S2 site, we incubated different cell lines with the metalloprotease inhibitor BB-94 pior to TFP stimulation, with the result that BB-94 diminished accumulation of P2 in cell lysates and concomitant reduced the amount of the shed fragments, E-subunit and P-stulk, in cell supernatants in 786-O, Caki-1 and COS7 cells (Fig. 15).

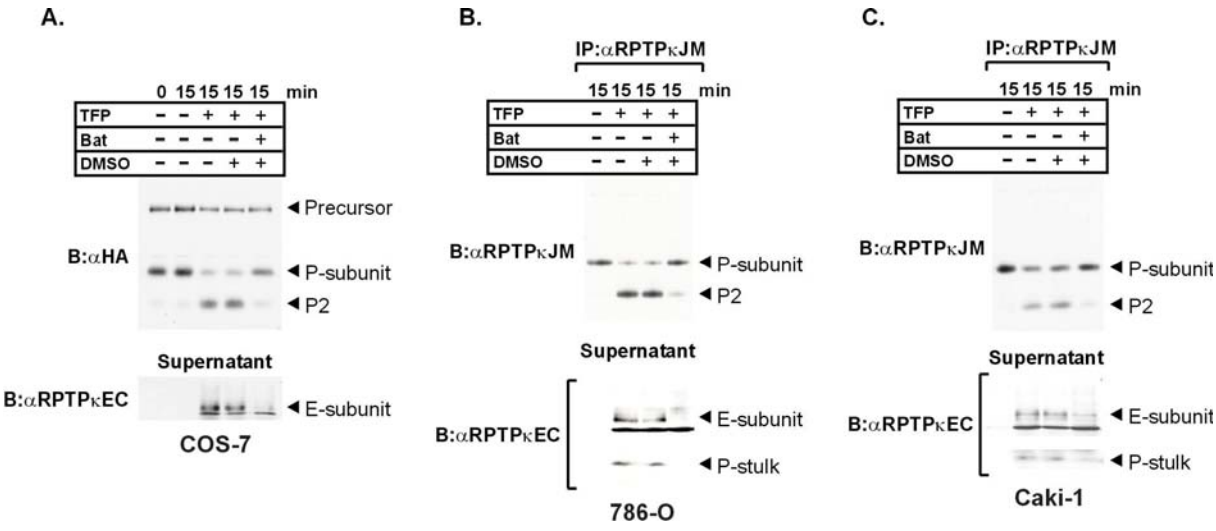

Figure 15: The metalloprotease inhibitor BB-94 diminishes S2 cleavage and shedding of κ in COS-7 (A), 786-O (B) and Caki-1 cells (C). *COS-7 cells were transfected with cDNA of RPTPκ-HA, whereas endogenously expressed κ was analysed in 786-O and Caki-1 cells. Prior to stimulation, cells were washed and serum-free medium was added. Cells were pretreated either with or without the metalloprotease inhibitor BB-94 (5μM) or DMSO and were then stimulated with 100μM TFP as indicated. Upper panel: Transfected κ was detected by antibody αHA in immunoblot analyses or endogenously expressed κ was immunoprecipitated with antibody αRPTPκJM and blotted as indicated. Lower panel: Conditioned media were collected, proteins concentrated by TCA-precipitation, blotted and probed with antibody αRPTPκEC.*

1.2.4. The S2 cleavage mechanism depends on the functionality of endocytosis that targets the S2 product for degradation

Certainly shedding of κ dimishes its ability to mediate homophilic binding. We therefore hypothesized that generation of P2 and subsequent shedding of the extracellular portion could be part of an internalization mechanism. If so, metalloprotease inhibitor treatment should prolong the κ's half life. We therefore performed pulse-chase experiments in MDA-MD-468 cells over an incubation time of 24 hours (Fig. 16). First of all, the estimated half-life of the enzyme was rather long (approximately 12 hours) and, importantly, it was significantly extended by addition of batimastat to cells in such a way that we could not observe any downregulation of the protein in a time window of 24 hours. We conclude that metalloprotease-mediated S2 cleavage is critically involved in regulation of the turnover of RPTPκ.

We hypothesized that generation of P2 is part of an internalization mechanism and analysed P2 production under conditions that are known to interfere with receptor internalization. For instance, cellular K^+depletion is a widely used technique to block receptor-mediated endocytosis by prevention of clathrin-coated pit formation (Zhou et al., 2001; Bayer et al., 2001; Sieczkarski et al., 2002). Moreover, cholesterol extraction from the

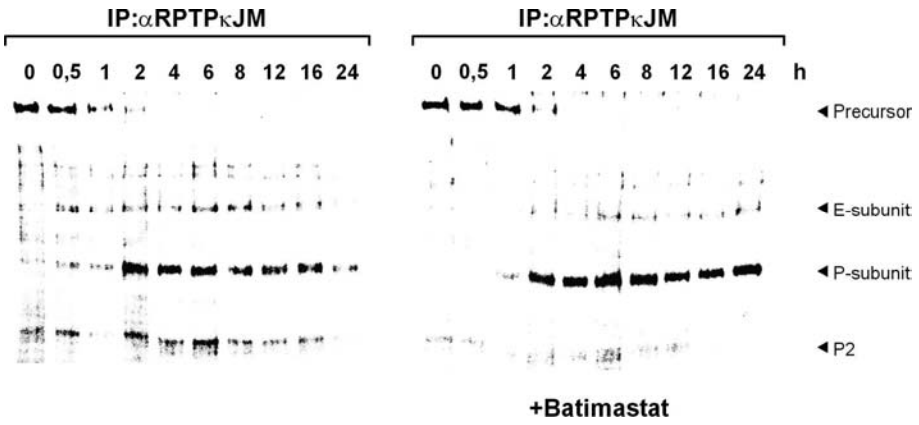

Figure 16: Metalloprotease inhibitor treatment decelerates κ turnover. *Pulse chase experiment in the absence and presence of the metalloprotease inhibitor batimastat. MDA-MB-468 cells were S^{35} labelled as described in "Materials and Methods" and incubated with chase-medium with or without Batimastat (5μM) for the time intervals indicated.*

plasma membrane by addition of methyl-ß-cyclodextrin (MßCD) was shown to inhibit both, clathrin-coated and caveolae-dependent endocytic vesicle formation (Hailstones et al., 1998; Rodal et al., 1999). Fig. 17 shows that both conditions actually diminish TFP-induced P2 formation. Also, basal cleavage at S2 is disturbed upon inhibition of clathrin-coated pit formation by using the K^+depletion method (upper panel). We proceeded to block clathrin-dependent endocytosis in a more specific approach, i.e. by ectopically expressing dominant-negative constructs of Eps15, an adaptor protein that actively participates in the formation of clathrin-coated pits where it is thought to be recruited to ubiquitinated plasma membrane receptors via its ubiquitin-interacting motif (UIM) and additionally to AP-2 via its C-terminal domain. Moreover, three Eps15 homology (EH) domains are required for Eps15 function. Several reports showed that transient expression of dominant-negative Eps15 lacking two EH domains (EΔ95/295) or, alternatively, expression of a construct that is devoid of all three EH domains (DIII) inhibit clathrin-coated pit assembly (Benmerah et al., 1998, 1999, 2000). Importantly, expression of EΔ95/295 and DIII do not interfere with S2 cleavage, demonstrating that clathrin-dependent internalisation is not involved in κ cleavage at S2. Thus, S2 processing of κ most likely proceeds via caveolae-dependent trafficking.

TFP was identified as antagonist of calmodulin function and it has been suggested that TFP treatment of cells leads to an increase in the concentration of cytosolic calcium (Chen et al., 2002). Initially, we asked whether the intracellular calcium chelator BAPTA-AM affects TFP-induced S2 processing. Indeed, preincubation of cells with BAPTA-AM lowers the extent κ cleavage, while BAPTA, a membrane-impermeable derivative, is not inhibitory (Fig. 18).

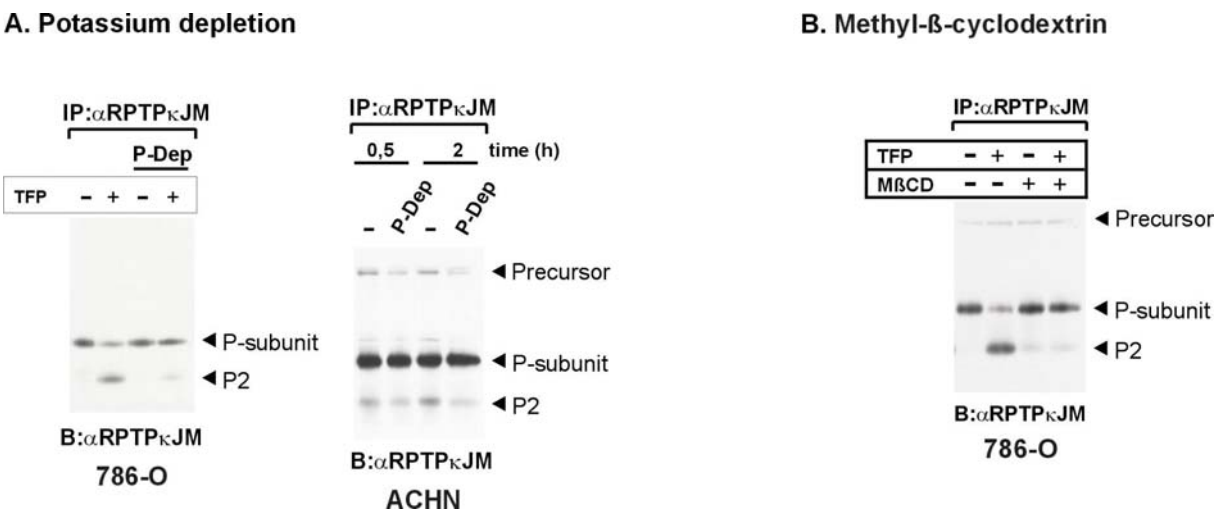

Figure 17: Conditions that block clathrin-dependent endocytosis diminish cleavage at S2. *Cellular potassium depletion: Left panel: 786-O cells were incubated with potassium depletion buffer (P-Dep) for 2h at 37°C, followed by incubations with regular medium or potassium depletion buffer in the presence or absence of 100µM TFP for 15 min at 37°C as indicated. Right panel: ACHN cells were exposed to potassium depletion buffer (P-Dep) for 2h at 37°C and then incubated with regular medium or potassium depletion buffer for further 0,5 or 2h as indicated. Methyl-ß-cyclodextrin (MßCD) treatment: 786-O cells were pretreated for 20min with or without 10mM methyl-ß-cyclodextrin (MßCD) and subsequently stimulated with 100µM TFP for 20min in the presence or absence of MßCD.*

However, it has become clear that intracellular calcium is actively involved in processes including exo- and endocytosis. So it is more likely that calcium chelation is just another way to block receptor internalisation and thus S2 cleavage.

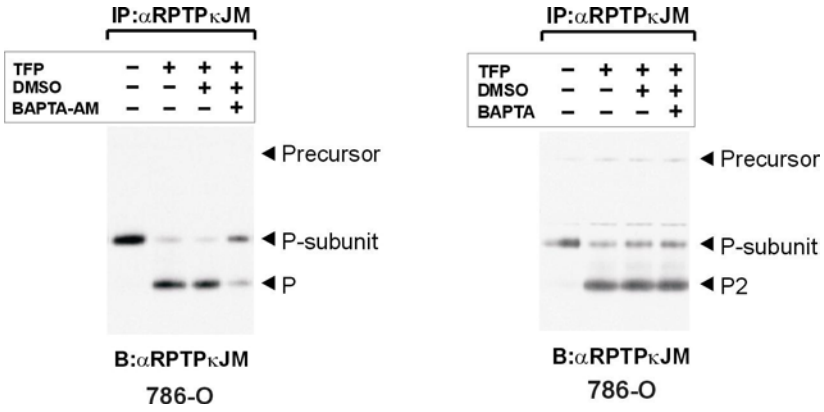

Figure 18: Inhibitory effect of BAPTA-AM, the membrane-permeable aminomethylester of BAPTA, on TFP-induced S2 processing. *Note that BAPTA (right) is not inhibitory. Cells were incubated for 2 hours with 10 µM of either BAPTA-AM or BAPTA. RPTPκ was immunoprecipitated and detected as indicated.*

It is well accepted that receptor-mediated endocytosis can result in lysosomal degradation of the cargo. In order to analyse if P2 is targeted for degradation, we tested inhibitors of lysosomal and proteasomal destruction (Fig. 19). MG132 caused accumulation of P2 while the proteasome inhibitor lactacystin had no effect (data not shown). Note that MG132

treatment only results in increase of the amount of P2, but not of the precursor or the P-subunit. In accord with our hypothesis ascribing a role of S2 cleavage in RPTPκ internalization and degradation, there was no difference in *in vitro* enzymatic activity of κ derived from TFP-treated or untreated cells (Fig. 20B). Moreover, transfection of either wild type receptor or a truncated version lacking the extracellular portion into 293-cells did not reveal any differences regarding to tyrosine phosphorylation of ß-catenin, a reported RPTPκ substrate (Fuchs et al., 1996) (data not shown).

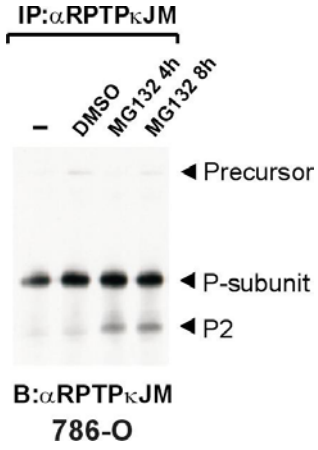

Figure 19: The proteasomal and lysosomal inhibitor MG132 causes P2 accumulation. *786-O cells were pretreated with 10μM MG132 for 4h and 8h or with DMSO. RPTPκ was immunoprecipitated and blotted with antibody αRPTPκJM.*

EGF stimulation induces association of EGFR with ß-Catenin in MDA-MB-468 mammary carcinoma cells, resulting in phosphorylation of ß-Catenin (Hazan and Norton, 1998). However, TFP treatment of MDA-MB-468 cells led to a strong decrease in EGF-induced tyrosine phosphorylation of ß-Catenin without affecting the activity of EGFR (Fig. 20A). Thus, decreased ß-Catenin tyrosine phosphorylation induced by TFP results from increased phosphatase activity. These contrasting findings could be explained by the fact that ß-Catenin dephosphorylating PTPs other than κ become activated upon TFP treatment.

Altogether, these results support a mechanism that requires initial steps of the clathrin-based endocytic pathway in order to accomplish metalloprotease-catalysed generation of P2, which in turn becomes degraded in lysosomes or proteasomes.

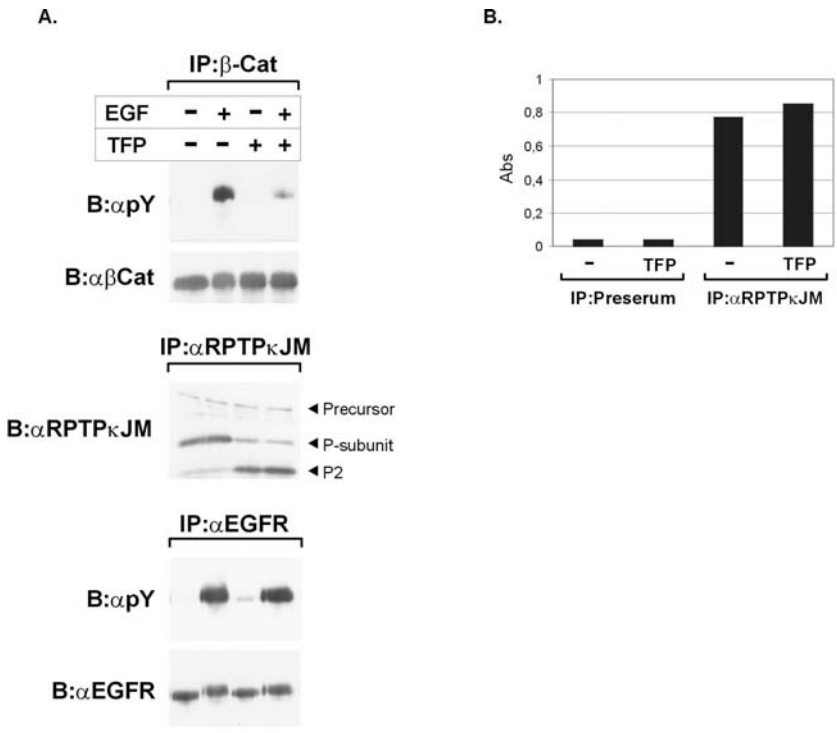

Figure 20: Effect of TFP treatment of MDA-MB-468 cells on RPTPκ catalytic activity in vivo and in vitro. *(A) Analysis of ß-Catenin tyrosine phosphorylation upon TFP treatment in the cellular context. Cells were incubated with TFP (100µM) for 10 minutes prior to stimulated with EGF (200ng/ml) for 5minutes as indicated. ß-Catenin (upper panel), RPTPκ (middle panel) and EGFR (lower panel) were immunoprecipited and analyzed. (B) MDA-MB-468 cells were treated with or without TFP for 30min, κ was immunoprecipitated from the cell lysate or preserum was added to the lysate as indicated. Phosphatase activity of immunoprecipitates was measured spectrophotometrically by using para-nitrophenyl-phosphate as substrate as described in "Materials and Methods".*

1.3. S3 processing of RPTPκ

1.3.1. Cleavage at site 3 generates the cytoplasmic isoform RPTPκ P3

Analysis of RPTPκ protein expression in human kidney tissue revealed the presence of an additional κ-specific isoform smaller than P2 (approximately 70kD) that we named P3 (κ cleavage product 3) (Fig. 21A, right lane). Paradoxically, we failed to detect P3 in renal carcinoma cell lines, like Caki-1. Also, TFP treatment did not induce P3 as shown for P2. However, when κ is ectopically expressed in HEK293 cells, P3 accumulation was observed under cell culture conditions. Moreover, immunoblot detection of the P3 C-terminus with HA antibody excluded the involvement of any C-terminal cleavage but instead confirmed that P3 generation must be the result of cleavage close to site 2 (Fig. 21B). This conclusion is also justified by the fact that we failed to detect alternatively spliced variants of κ in northern blot analysis performed from 36 kidney tissue samples that accumulated the P3 protein fragment as shown by immunoblot analysis (see below).

Since S3 cleavage proceeds downstream of site 2, we expected that P3 is exclusively localized in the cytosol. To experimentally confirm our model, we investigated subcellular localization of P3 by membrane fractionation of human kidney tissue extracts. As Fig 22 shows, P3 is indeed a cytoplasmic, two PTP domain-containing κ isoform. We conclude that cleavage at S3 leads to relocation of κ-PTP domains from the membrane environment into the cytoplasm.

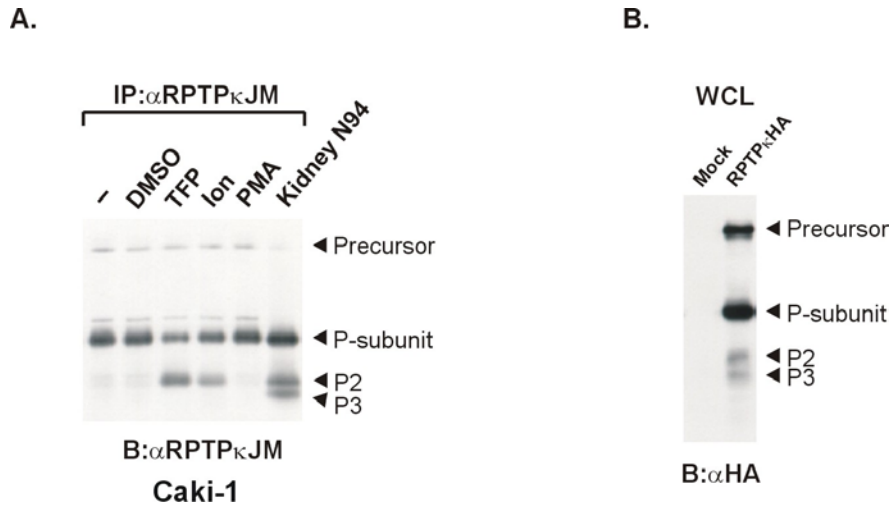

Figure 21: An additional, truncated isoform (P3) accumulates in human kidney tissue and HEK293 cells ectopically expressing RPTPκ. *(A) Caki-1 renal carcinoma cells were treated with 1μM of the phorbolester PMA, 1μM of ionomycin (Io) and 100μM trifluoperazine (TFP) for 30 min to induce P2. For comparison, RPTPκ was immunoprecipitated from human kidney tissue using αRPTPκJM antibody to the intracellular juxtamembrane part. (B) Ectopically expressed RPTPκ, HA-tagged at the C-terminus, was detected in HEK293 cells with αHA antibody to visualize the intact C-terminus of P3.*

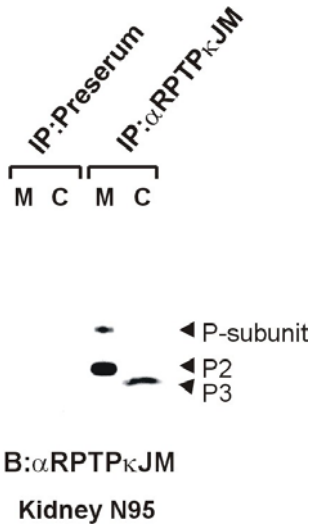

Figure 22: P3 is exclusively localized in the cell cytoplasm. *Human kidney tissue material was prepared as described in "Materials and Methods". Soluble, cytosolic (C) and membrane-bound proteins (M) were separated by membrane fractionation and RPTPκ was immunoprecipitated from both fractions by using antibody αRPTPκJM.*

1.3.2. Presenilin 1 mediates S3 processing of RPTPκ

Stimulation of the Notch receptor by the Dsl ligand on a neighbouring cell induces S2 cleavage and shedding of Notch mediated by ADAM10 or TACE, which in turn leads to the next cleavage (S3) by presenilin within the plane of the plasma membrane. This in turn releases the intracellular fragment of the receptor (Fig. 6). Importantly, the S2 site was identified 12 amino acids amino-terminal of the TM region, and S3 cleavage targets amino acid V1744 in the midle of the TM region. Thus, both C-terminal cleavage fragments differ in size by only 22 residues. So, Presenilin is a good candidate protease that may mediate processing of κ at site 3.

We therefore analysed processing of endogenous and ectopically expressed κ in HEK293 cells stably expressing Presenilin 1 wt or a dominant-negative version, characterized by substitution of the catalytic aspartate to asparagine. As Fig. 23 shows, fragment P3 (since it is the product of cleavage at site 3) accumulates in cells in which endogene Presenilin function is blocked by expression of the dominant-negative version, but not in cells expressing the wt protease. We conclude that the metalloprotease-generated S2 produkt, but not the P-subunit, is further processed by Presenilin 1, a unique protease that directly releases the cytoplasmic fragments of cell surface receptors by cleavage within their transmembrane regions.

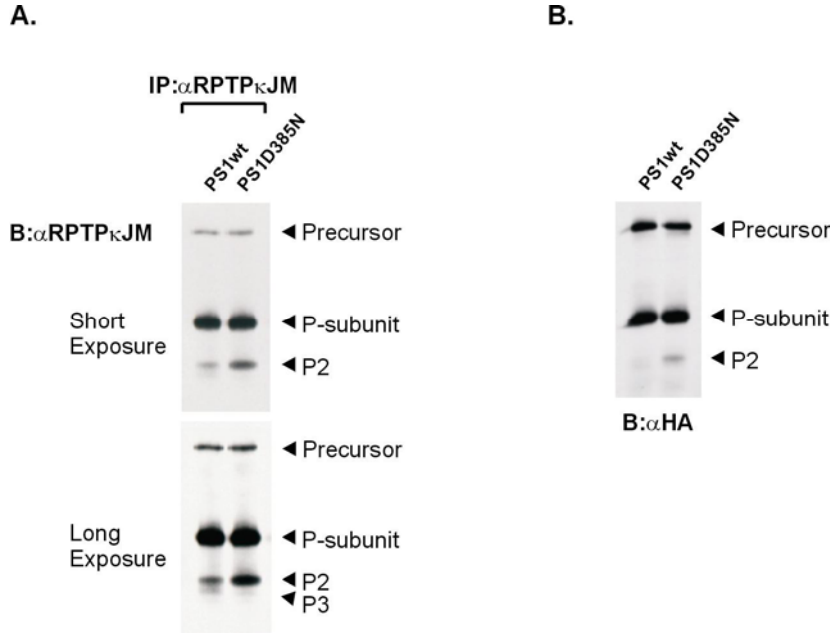

Figure 23: Inhibition of endogenous Presenilin 1 (PS1) causes accumulation of the RPTPκ S2 product and a decrease in P3. *(A) Endogenous RPTPκ was immuno-precipitated with antibody αRPTPκJM from cell clones stably expressing either PS1 wild-type or a dominant-negative version in which the catalytic aspartate was substituted by asparagine. (B) Ectopically expressed RPTPκ-HA was analysed in whole cell lysate as indicated.*

1.3.3. P3 accumulation is diminished in primary human renal carcinomas

Some groups have reported downregulation of κ expression in melanoma cell lines and lymphomas (McArdle et al., 2001; Nakamura et al., 2003). We therefore analysed the expression of the κ protein isoforms and mRNA in kidney tumor tissue (T) and surrounding normal kidney tissue (N) derived from 25 patients which underwent nephrectomy. κ was immunoprecipitated from tissue samples and κ-specific signals obtained from western blots were quantified. Moreover, RPTPκ mRNA expression was investigated by northern blot analysis and corrected with expression of the housekeeping gene ß-actin (Fig. 24A, B).

To numeralize the extent of downregulation of κ protein isoforms, including the P-subunit, P2 and P3, as well as mRNA in tumor samples relative to the normal, surrounding tissues, we calculated the quotients of $(T/N)_{RPTP\kappa}$ and $(T/N)_{Tubulin}$ or $(T/N)_{RPTP\kappa}$ and $(T/N)_{ß\text{-}Actin}$ and expressed them as decad logarithm as shown in Fig. 25. Thus, values that fall below -1 correspond to a relative downregulation by > 90%. By using this approach, we observed specific decreases in the expression of κ protein isoforms, i.e. for the P-subunit: 3 out of 25 samples, P2: 5 out of 25 samples and P3: 10 out of 25 samples are downregulated by > 90% when compared to normal surrounding tissue (Tab. 5). Thus, κ isoform expression is specifically decreased with strongest repression of P3.

Analysis of relative κmRNA expression revealed no significant downregulation, i.e. only 1 out of 18 samples showed a decrease in expression by >90% (Fig. 25B, Tab. 5). In accordance with previous data presented in this work, we could not detect alternative κ-specific messengers, underscoring the fact that P2 and P3 are proteolysis-derived isoforms in human tissue. Taken together, the results point to a tumor suppressive function of P3, the final product of a proteolytic cascade involving processing at S1, S2 and S3 in progression of primary renal carcinoma.

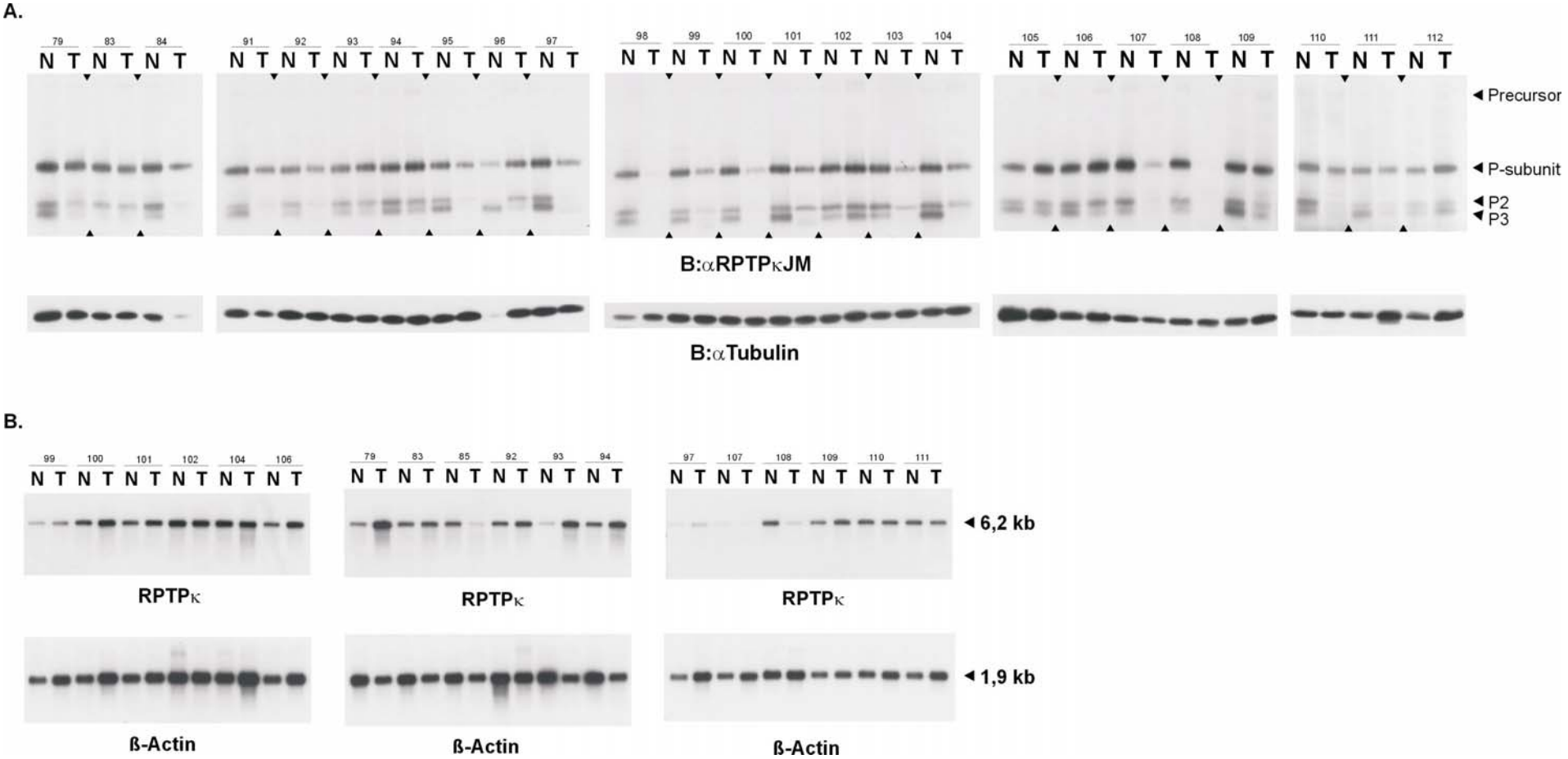

Figure 24: Analysis of RPTPκ protein (A) and mRNA (B) in human kidney tumor tissue (T) and surrounding normal kidney tissue (N) derived from 25 patients which underwent nephrectomy. *(A) Tissue material was powderized under liquid nitrogen conditions and processed for RPTPκ immunoprecipitation by using αRPTPκJM antibody or protein lysates were subjected to SDS-PAGE and probed with αtubulin antibody. (B) Total RNA, Poly(A)$^+$ RNA, and cDNA probes were generated as described in "Materials and Methods". Northern blot analysis for expression of the RPTPκ gene in kidney tissue samples was performed as described. Blots were hybridized with RPTPκ sequence 2238-4317. Loading of mRNA samples was verified by rehybridization of filters with a human β-actin probe.*

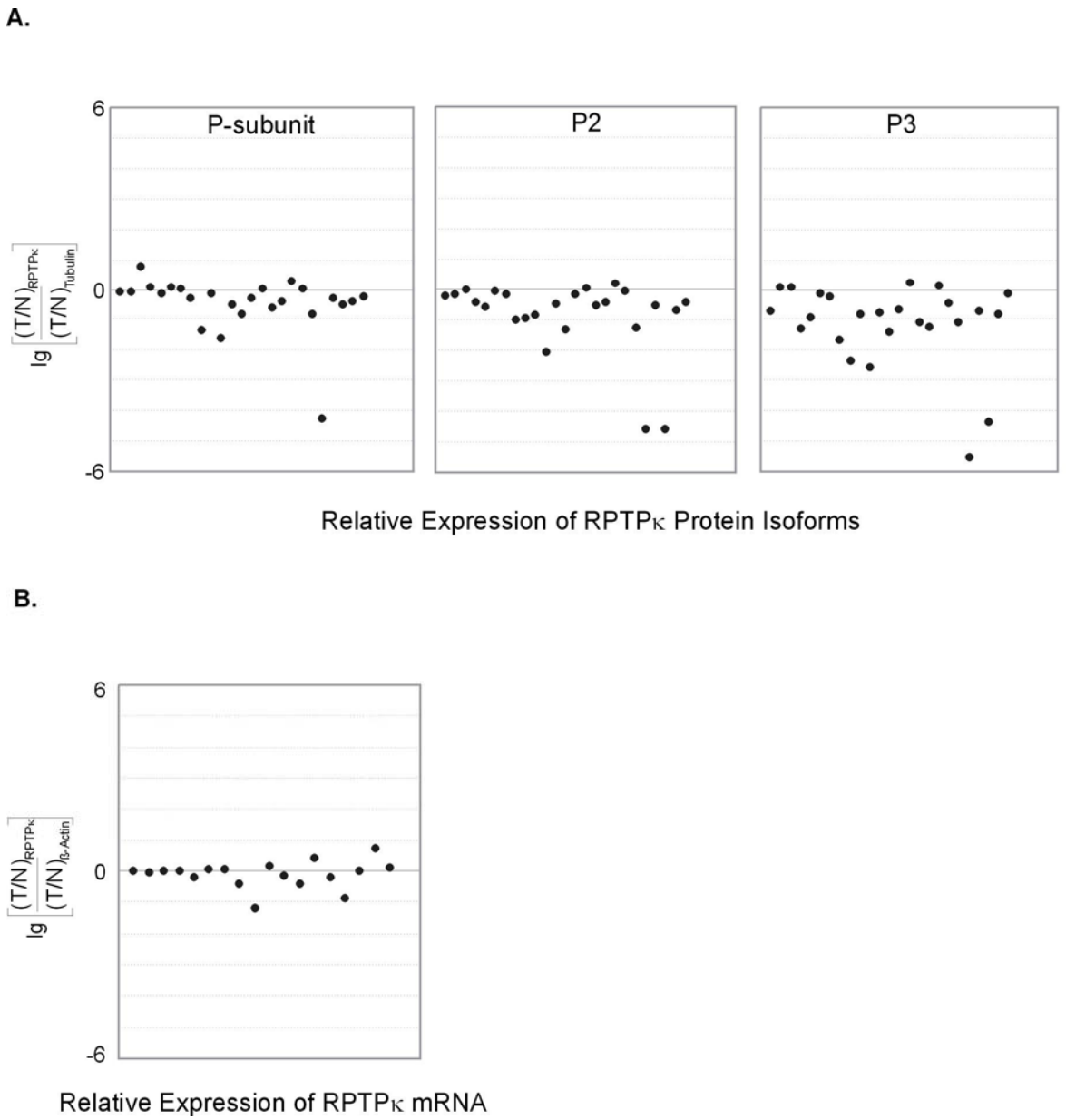

Figure 25: Relative expression of RPTPκ protein isoforms (A) and mRNA (B) calculated from data shown in Fig. 24*. Blots were analysed by scanning densitometry and relative expression (R) calculated according to equation* $R = \log_{10} ((T/N)_{RPTP\kappa} / (T/N)_{\beta\text{-}Actin})$*. For instance, values <-1 correspond to a downregulation >90%. Note that the relative abundance of P3 is decreased in 10 out of 25 samples by more than 90%, whereas the P-subunit expression is reduced in only 3 samples by the same extent.*

RPTPκ mRNA	RPTPκ 100	RPTPκ 75	RPTPκ70
1/18	3/25	5/25	10/25
5,6%	12%	20%	40%

Table 5: Relative downregulation of RPTPκ mRNA and protein isoforms (number of samples with >90% downregulation/number of total samples). *The data were obtained from Fig. 25.*

2. Antibody αRPTPκ EC as κ "pseudoligand"

A major obstacle in the field of RPTP research is the lack of cognate ligands with the ability to modulate RPTP activity. For instance, by means of such ligands RPTP signaling could be switched on or off so that RPTP substrates could be easily identified.

The antibody αRPTPκ EC, produced in the course of this work and raised against the extracellular part of the enzyme, was found to be an appropriate tool for studying the function of κ in the cellular context. We hypothesized that such an antibody, when supplemented in cell supernatants, could induce aggregation of the endogenously expressed receptor-like phosphatase at the cell surface, thereby activating or inhibiting the activity of the enzyme, depending on whether aggregation leads to RPTP activation or inhibition.

ß-Catenin was suggested to serve as substrate for κ since physical interaction between κ and ß-Catenin was demonstrated in HT-29 colon carcinoma cells by coimmunoprecipitation as well as GST pulldown assays and the juxtamembrane part of the phosphatase was identified as ß-catenin binding site (Fuchs et al, 1996). Moreover, some amino acid residues within the juxtamembrane sequences of κ and Cadherins are conserved, indicating that both receptors make use of a similar mechanism in order to bind to ß-catenin. However, dephosphorylation of ß-catenin by RPTPκ in the cellular context was not yet demonstrated.

We have analysed ß-catenin tyrosine phosphorylation upon stimulation of MDA-MB-468 cells with EGF in the presence or absence of RPTPκ EC-specific antibody in the cell medium. Fig. 26A shows that antibody pretreatment lowers the extent of ß-catenin tyrosine phosphorylation, pointing to a mechanism of antibody-induced RPTP aggregation and activation. The effect provoked by antibody treatment was shown to dose-dependent (Fig. 26B). Intriguingly, when analyzing the RPTPκ protein, we observed that the antibody provoked rapid cleavage of κ (probably at S2) (Fig. 27). Importantly, antibody-induced processing is not inhibited by metalloprotease inhibitors, indicating the involvement of other proteases or other processing events like S3, respectively (data not shown).

Next we asked whether the catalytic activity of the membrane-proximal PTP domain is mediating dephosphorylation of ß-catenin. To this end, we cotransfected HEK293 cells with a constitutive active mutant of src (to induce tyrosine phophorylation of ß-catenin), in which

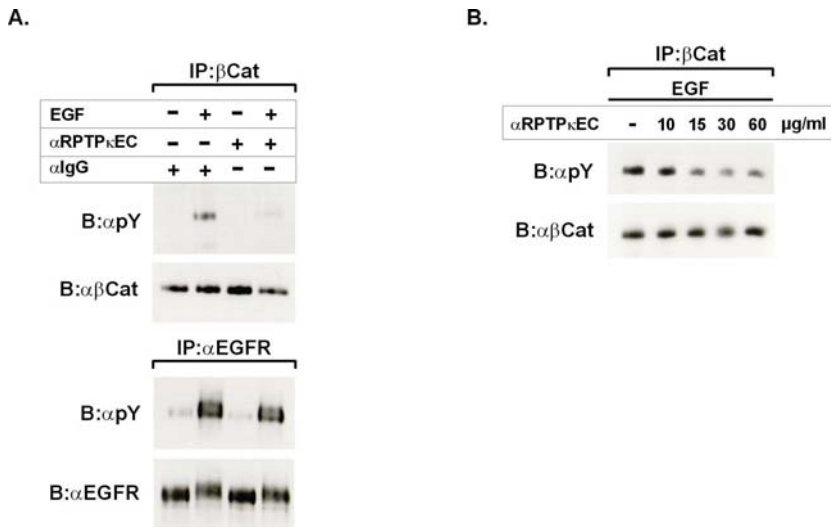

Figure 26: αRPTPκEC antibody treatment of MDA-MB-468 cells leads to decreased tyrosine phosphorylation of ß-Catenin, a κ substrate. *(A) Cells were incubated with RPTPκEC-specific antibody (30µg/ml) for 30min prior to stimulated with EGF (200ng/ml) for 5minutes as indicated. IgG antibody was used as control. (B) Reduction in ß-Catenin tyrosine phosphorylation is antibody concentration-dependent. Cell stimulation was performed with varying concentrations of antibody as indicated.*

tyrosine 529 was replaced by phenylalanine (scrYF), and either RPTPκ or RPTPκ-C/S_{prox}, harbouring a transistion of the membrane-proximal catalytic cysteine to serine. Fig. 28 shows that src-induced ß-Catenin tyrosine phosphorylation is decreased in the presence of RPTPκ, but not of RPTPκ-C/S_{prox}. We conclude that the catalytic activity of the membrane-proximal PTP domain is required for dephosphorylation of ß-catenin in the cellular context.

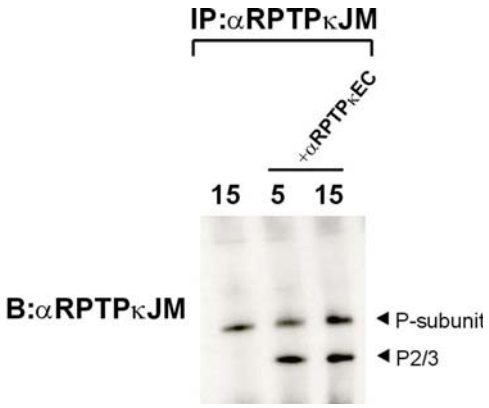

Figure 27: Antibody αRPTPκEC treatment of cells leads to rapid cleavage of endogenously expressed RPTPκ, generating P2/3. *MDA-MB-468 cells were treated with αRPTPκEC antibody for 30min. RPTPκ was immunoprecipitated and analysed with antibody αRPTPκJM. Since the cleavage is not BB-94-sensitive, we can not exclude the involvement of S3 cleavage or other processing events.*

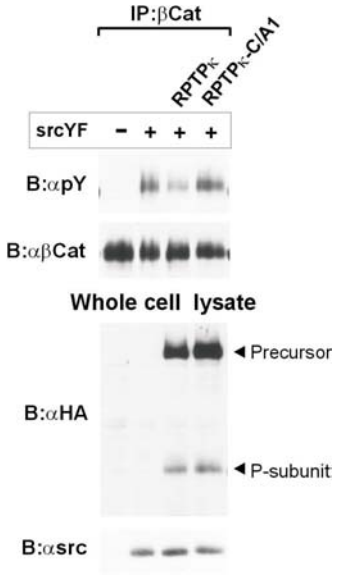

Figure 28: ß-Catenin dephosphorylation depends on the catalytic activity of the membrane-proximal PTP domain of κ. *HEK293 cells were cotransfected with a constitutive activ mutant of src (srcYF) and either RPTPκ-HA or RPTPκ-C/A$_{prox}$HA. Cells were starved for 12h and ß-Catenin was immunoprecipitated and probed with 4G10 αpY antibody (upper panel). Whole cell lysate was analysed for expression of RPTPκ and srcYF.*

3. Plasminogen as RPTPκ MAM domain binding protein

3.1. Plasminogen binds to the MAM domain of RPTPκ

As mentioned earlier, MAM-family RPTPs undergo homophilic binding. Several groups have reported that this binding is highly specific, i.e. RPTPκ proteins only interact with RPTPκ proteins expressed at the surface of an adjacent cell and there is no heterophilic binding between, for instance, RPTPκ and RPTPμ. By using fusion constructs of both receptors in cell adhesion assays performed with baculovirus-infected SF9-cells, involvement of both, the MAM-domain and the Immunglobulin domain in homophilic binding was demonstrated. Moreover, it was suggested that the MAM domain controls the specificity of such *trans* interactions. However, some receptors that undergo homophilic binding have also been shown to bind in addition to heterophilic, soluble ligands. For example, the extracellular fragments of the protein tyrosine kinase Axl bind to each other in *trans*, but they also serve as docking sites for their ligand Gas-6, respectively.

Only few soluble ligands for RPTPs have been reported. Although RPTPκ is a potential cell contact receptor in epithelial cells, the enzyme is also expressed in lymphocytes, but not in endothelial cells. This observation may suggest that RPTPκ serves a sensor function other

than that mentioned above. For instance, in analogy to the RTK Axl, κ could be a receptor for a serum-derived protein.

The aim of this part was therefore the identification of potential soluble ligands for RPTPκ. To this end, we expressed several parts of the extracellular fragment of the phosphatase, fused to GST, in HEK293 cells. A scheme of the MAM domain GFP fusion protein used in this experiment is shown in Fig. 29A. Since the fusion protein harbours a N-terminal signal sequence and is devoid of its transmembrane region, it becomes secreted into the cell culture medium. For purification, cell supernatants were applied to a GSH-Sepharose column and following addition of gluthation we observed coelution of a protein of approximately 90kD (Fig. 29B). Internal EDMAN sequencing identified the protein as bovine Plasminogen (Fig.

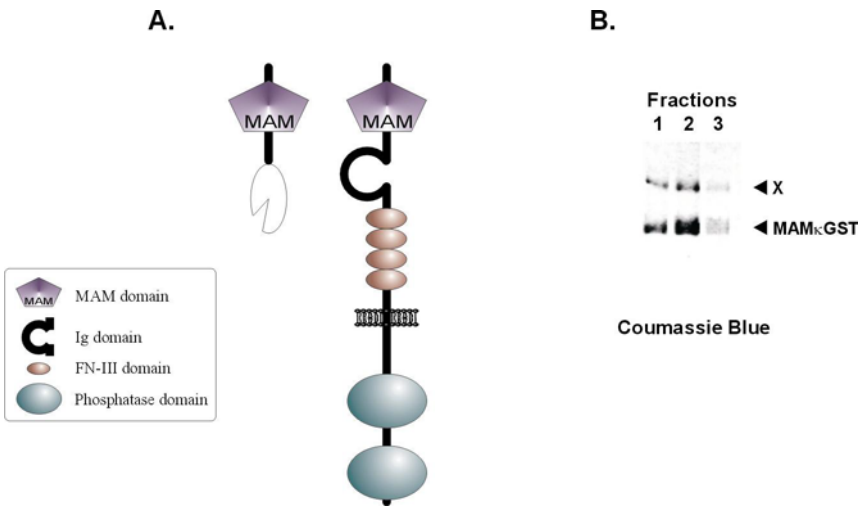

Figure 29: Coelution of protein X with RPTPκ-MAM-GST in a GST-pulldown assay. *(A) Schematic representation of RPRPκMAM-GST domain structure. GST was fused to the C-terminus of the MAM domain of RPTPκ. (B) SDS-PAGE analyses of fractions obtained following elution of RPRPκMAM-GST with glutathione. Shown are the first three fractions. Cell supernatants (DMEM, 10% FCS) obtained from RPTPκ MAM-GST-secreting HEK293 cells were applied to the GSH-Sepharose column. The column was washed with PBS and the protein eluted with Glutathion-containing Tris buffer. Note coelution of protein X of approximately 90kD.*

30). This finding suggests that FCS-derived Plasminogen binds to the MAM-domain of human RPTPκ. To control specificity of binding, we applied the same experimental design to a panel of GST fusion proteins, namely RPTPκ EC-GST, RPTPκ MAM-GST, RPTPμ EC-GST and RPTPμ MAM-GST. The proteins were purified from FCS-containing media with a GSH-Sepharose column and the fractions analysed by western blotting with Plasminogen-specific antibody (Fig. 31). By using this approach, Plasminogen was detected in the RPTPκ MAM-GST-containing fraction, but not in that containing RPTPμ MAM-GST or RPTPκ EC-GST, indicating specificity in binding. Given the overall hydrophobicity of

Plasminogen, we proceeded with covalent immobilization of the protein onto CNBr-Sepharose beads. The different GST-fusion proteins were applied to beads and after extensive washing, beads were boiled with Lämmli and the probes analyzed by western blot with GST-specific antibody (Fig. 31B). In this invers binding assay, BSA was additionally

```
                                    DLLDDYVNTGGASL
BOVINE     1   MLPASPKMEHKAVVFLLLLFLKSGLGDLLDDYVNTQGASLLSLSRKNLAGRSVEDCAAKC   60
HUMAN      1   -------MEHKEVVLLLLLFLKSGQGEPLDDYVNTQGASLFSVTKKQLGAGSIEECAAKC   53
```

Figure 30: Internal EDMAN-sequencing analysis identified Plasminogen as coeluting protein. *Alignment of Plasminogen N-terminal sequences from bovine and human. The sequence obtained from internal EDMAN-analysis is aligned. Before analysis, the protein sample was digested with endoproteinase Gly-C to avoid interference with acetylated protein N-termini.*

immobilized to beads (negative control) and GSH-Sepharose was used to quantify the input of the fusion proteins (positive control). These results revealed binding of Plasminogen to RPTPμ MAM-GST as well, however, the bindung was weak and a larger quantity of the protein was applied to beads (Fig. 31B). In accord with this observation, quantification of protein bands revealed that approximately 70% of input of RPTPκ MAM-

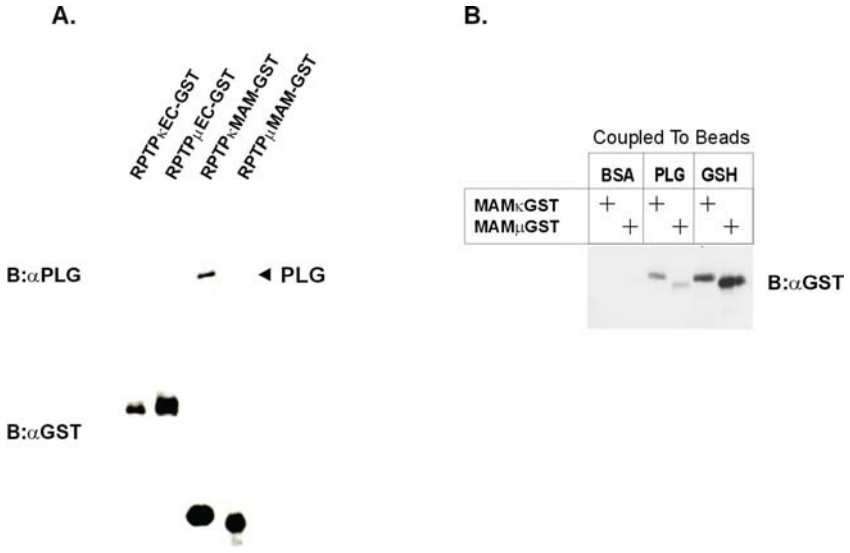

Figure 31: Binding of Plasminogen to RPTPκMAM-GST. *(A) 10% FCS-containing cell supernatants derived from GST-fusion protein secreting HEK293 cells were applied to GSH-Sepharose beads, the beads were washed and boiled with Lämmli. Probes were analyzed by western blotting with αGST antibody. Note that Plasminogen is detected in the RPTPκMAM-GST sample, but not in those containing RPTPκEC-GST or RPTPμMAM-GST. (B) Invers Plasminogen-binding assay. BSA beads were used as negative control. BSA and human Plasminogen were immobilized to CNBr sepharose beads as described in "Materials and Methods". GSH beads were used to quantify the total input of secreted MAM-GST fusion proteins. 1ml of cell supernatans derived from fusion protein-secreting HEK293 cells were incubated with beads end-over end for 16h. Beads were washed, boiled with Lämmli and analyzed by western blotting with GST-specific antibody.*

GST associates with Plasminogen, whereas the binding of RPTPμ MAM-GST was reduced to 10% when compared to the respective protein input. Together these experiments show that the Plasmin precursor Plasminogen preferentially associates with the MAM domain of κ.

3.2. Plasminogen recognizes a palindromic sequence

To determine the binding motif within the MAM domain sequence, we screened cellulose-bound peptide scans (Reineke et al., 2001) representing the complete sequence of the MAM domain comprising 178 residues for Plasminogen binding. This approach is justified by the fact that Plasminogen was previously assayed for binding to a synthetic peptide derived from the bacterial surface protein PAM (Schenone et al., 2000). The peptide scans were composed of 13mers that overlap with adjacent peptides by eleven residues (Fig. 32A). The library was incubated with human Plasminogen to equilibrium, followed by electrotransfer and immunodetection of bound Plasminogen. The peptide scan shows that Plasminogen associates only with two adjacent peptides characterized by the consensus sequence DFSYLLYSQKG (Fig. 32B). Interestingly, the motif contains a six residue palindrom which is not found in RPTPμ (Fig. 32C), probably explaining the fact that binding of its MAM domain is much weaker as compared to the one of κ. Moreover, the binding motif contains a lysine residue and several reports have demonstrated the importance of lysine residues in Plasminogen binding (Ranson et al., 1998; Andronicos et al., 2001).

To investigate the contribution of the different residues to Plasminogen binding, we performed substitutional analysis of the identified binding sequence. Fig. 33 shows that aspartate at postion one and lysine at position ten are absolutely required for Plasminogen binding. However, the ability to bind Plasminogen is retained in case of substitution of both residues by equally charged amino acids, i.e. glutamate and arginine. Thus, the negative charge at position one and the positive charge at position ten seem to be involved in binding of RPTPκMAM to Plasminogen. Additionally, substitution of the two tyrosine residues contained within the palindromic sequence diminished binding, but again, the binding is partially restored by replacement with phenylalanine or other hydrophopic residues like leucine, valine or isoleucine.

In order to identify the minimal binding sequence we performed a so called length scan by synthesizing truncated versions of the cellulose-bound peptide identified in Fig. 34.

A.

Sequences of 13mer peptides

1	FSAGGCTFDDGPG	43	QKGLNPGTLNILV
2	AGGCTFDDGPGAC	44	GLNPGTLNILVRV
3	GCTFDDGPGACDY	45	NPGTLNILVRVNK
4	TFDDGPGACDYHQ	46	GTLNILVRVNKGP
5	DDGPGACDYHQDL	47	LNILVRVNKGPLA
6	GPGACDYHQDLYD	48	ILVRVNKGPLANP
7	GACDYHQDLYDDF	49	VRVNKGPLANPIW
8	CDYHQDLYDDFEW	50	VNKGPLANPIWNV
9	YHQDLYDDFEWVH	51	KGPLANPIWNVTG
10	QDLYDDFEWVHVS	52	PLANPIWNVTGFT
11	LYDDFEWVHVSAQ	53	ANPIWNVTGFTGR
12	DDFEWVHVSAQEP	54	PIWNVTGFTGRDW
13	FEWVHVSAQEPHY	55	WNVTGFTGRDWLR
14	WVHVSAQEPHYLP	56	VTGFTGRDWLRAE
15	HVSAQEPHYLPPE	57	GFTGRDWLRAELA
16	SAQEPHYLPPEMP	58	TGRDWLRAELAVS
17	QEPHYLPPEMPQG	59	RDWLRAELAVSSF
18	PHYLPPEMPQGSY	60	WLRAELAVSSFWP
19	YLPPEMPQGSYMI	61	RAELAVSSFWPNE
20	PPEMPQGSYMIVD	62	ELAVSSFWPNEYQ
21	EMPQGSYMIVDSS	63	AVSSFWPNEYQVI
22	PQGSYMIVDSSDH	64	SSFWPNEYQVIFE
23	GSYMIVDSSDHDP	65	FWPNEYQVIFEAE
24	YMIVDSSDHDPGE	66	PNEYQVIFEAEVS
25	IVDSSDHDPGEKA	67	EYQVIFEAEVSGG
26	DSSDHDPGEKARL	68	QVIFEAEVSGGRS
27	SDHDPGEKARLQL	69	IFEAEVSGGRSGY
28	HDPGEKARLQLPT	70	EAEVSGGRSGYIA
29	PGEKARLQLPTMK	71	EVSGGRSGYIAID
30	EKARLQLPTMKEN	72	SGGRSGYIAIDDI
31	ARLQLPTMKENDT	73	GRSGYIAIDDIQV
32	LQLPTMKENDTHC	74	SGYIAIDDIQVLS
33	LPTMKENDTHCID	75	YIAIDDIQVLSYP
34	TMKENDTHCIDFS	76	AIDDIQVLSYPCD
35	KENDTHCIDFSYL	78	DDIQVLSYPCDKS
36	NDTHCIDFSYLLY	79	IQVLSYPCDKSPH
37	THCIDFSYLLYSQ	80	VLSYPCDKSPHFL
38	CIDFSYLLYSQKG	81	SYPCDKSPHFLRL
39	DFSYLLYSQKGLN	82	PCDKSPHFLRLGD
40	SYLLYSQKGLNPG	83	DKSPHFLRLGDVE
41	LLYSQKGLNPGTL	84	SPHFLRLGDVEVN
42	YSQKGLNPGTLNI	85	PHFLRLGDVEVNA

B.

38 CIDFSYLLYSQKG
39 DFSYLLYSQKGLN

C.

```
PTPk_MAM     1 FSAGGCTFDDGPGACDYHQDLYDDFEWVHVSAQEPHYLPPEMPQGSYMIVDSSDHDPGEK 60
                 :*** **:  .:*.* *.  ***:* :*.:       * **.** *:*::*.:  *::
PTPm_MAM     1 TFSGGCLFDEPYSTCGYSQSEGDDFNWEQVNTLTKPTSDPWMPSGSLMLVNASGRPEGQR 60

PTPk_MAM    61 ARLQLPTMKENDTHCIDFSYLLYSQKGLNPGTLNILVRVNKGPLANPIWNVTGFTGRDWL 12C
               *:* ** :********** *:: *:..  ** **: *:**:***.*****::* . * *
PTPm_MAM    61 AHLLLPQLKENDTHCIDFHYFVSSKSNSPPGLLNVYVKVNNGPLGNPIWNISGDPTRTWN 12C

PTPk_MAM   121 RAELAVSSFWPNEYQVIFEAEVSGGRSGYIAIDDIQVLSYPCDKSPHFLRLGDVEVNA 178
               *****:*:**** ******. .** :.**:***:::**.:** ::*****: :*****
PTPm_MAM   121 RAELAISTFWPNFYQVIFEVITSG-HQGYLAIDEVKVLGHPCTRTPHFLRIQNVEVNA 177
```

Figure 32: Mapping of the Plasminogen binding site on the RPTPκMAM domain. *(A) Overlapping peptides from the MAM domain bound to continuous cellulose membrane support. (B) Immunodetection of bound plasminogen. Purified human Plasminogen was assayed for binding to the MAM domain-derived peptide scanning library. Bound Plasminogen was blotted onto nitro-cellulose membrane and detected by using a Plasminogen-specific antibody. (C) Alignment of the MAM domains of κ and μ. The binding sequence identified in B is marked by bold letters. Note that the palindromic sequence ist not present in μ.*

Truncations of the N-terminus are not tolerated, indicating that the glutamate residue is required for binding to Plasminogen. In contast, successive deletion of the C-terminal GLN sequence does not interfere with binding, but removal of lysine completely abolished it. Thus, the 10mer sequence DFSYLLLYSQK, characterized by the presence of a central, hydrophobic, 6mer palindrome, was identified as minimal Plasminogen binding sequence.

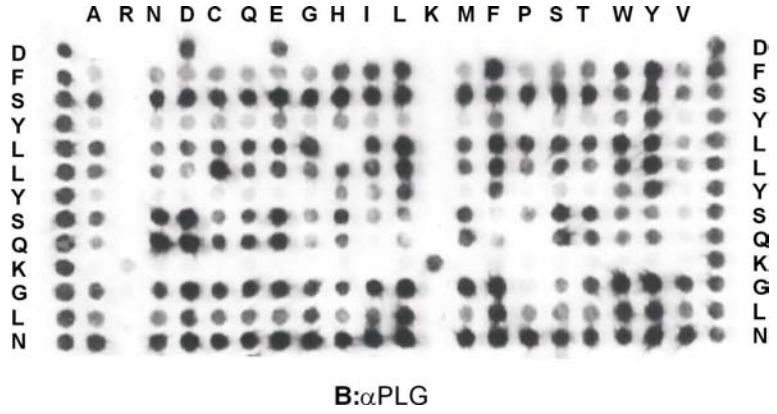

Figure 33: Identification of residues critical for Plasminogen binding by substitutional analysis of the peptide DFSYLLLYSQKGLN. *Each of the 13 residues contained within the peptide was substituted by all other 20 L-amino acids and analyzed for Plasminogen binding by immunodetection of bound Plasminogen after protein transfer to a nitrocellulose membrane. The sequences corresponding to the left and right columns of the library are identical and represent the starting peptide. All other spots are single substitution analogs. Note that the residues D (aspartate) at position 1 and K (lysine) at position 10 are required for binding and can only be substituted with equally charged residues. Moreover, substitution of tyrosine residues (Y) contained within the palindromic sequence leads to strong reduction in binding to Plasminogen, respectively.*

1	**DFSYLLYSQKGLN**	23	YLLYSQK
2	**DFSYLLYSQKGL**	24	LLYSQKG
3	FSYLLYSQKGLN	25	LYSQKGL
4	**DFSYLLYSQKG**	26	YSQKGLN
5	FSYLLYSQKGL	27	DFSYLL
6	SYLLYSQKGLN	28	FSYLLY
7	**DFSYLLYSQK**	29	SYLLYS
8	SYLLYSQKGL	30	YLLYSQ
9	YLLYSQKGLN	31	LLYSQK
10	DFSYLLYSQ	32	LYSQKG
11	FSYLLYSQK	33	YSQKGL
12	SYLLYSQKG	34	SQKGLN
13	YLLYSQKGL	35	DFSYL
14	LLYSQKGLN	36	FSYLL
15	FSYLLYSQ	37	SYLLY
16	SYLLYSQK	38	YLLYS
17	YLLYSQK	39	LLYSQ
18	LLYSQKGL	40	LYSQK
19	LYSQKGLN	41	YSQKG
20	DFSYLLY	42	SQKGL
21	FSYLLYS	43	QKGLN
22	SYLLYSQ		

Figure 34: Definition of the core binding sequence. *43 peptides sequences were tested for Plasminogen binding (top). Alignment of the synthesized truncated peptides is shown below. Note that both, the N-terminal glutamate and the C-terminal lysine, are required for binding.*

Since the identified Plasminogen binding sequence or related motifes have not been described, we conducted a similarity search with the sequence DFSYLLYSQKG by using the alignment tool *Blast* (www.expasy.org/tools/blast/). Protein sequences were extracted from databases Swiss-Prot/TrEMBL/TrEMBL_NEW (complete) using the PAM30 matrix with a treshold value of 100. Only sequences having seven out of eleven residues in common with DFSYLLYSQKG and which are characterized by the presence of the two central tyrosine residues within the palindrome were accepted. Under these conditions, only five protein sequences, that are not RPTP-derived, were obtained (Fig. 35). Intriguingly, all sequences are encoded by bacteria that are pathogenic, either to humans (*Bacteroides thetaiotaomicron, Helicobacter pylori, Yersinia pestis*) or to animals (*Plasmodium yoelii yoelii, Pasteurella multicoda*). Three sequences are solely based on expressed sequence tags (ESTs) while the functions of the other two proteins, PFEMP3 and yfeE, have been described (Waterkeyn et al., 2000; Glenister et al., 2002; Bearden and Perry, 1999). To date, none of these proteins has been implicated in binding to Plasminogen.

Human	RPTPk	PTMKENDTHCIDFSYLLYSQKGLNPGTLNILVRV
Bacteroides thetaiotaomicron	BT3853[2]	RVIADDSSCFDFISYLLYSRFNIVLTDKDRVIKN
Plasmodium yoelii yoelii	PY06049[3]	QSKSSILNNYKTLSYLLYSQKWDNNIIYDIINNS
Pasteurella multicoda	PM0507[4]	LAILFSNLLVFLFAYLLYRQKTRIKSFNFRQYKT
Helicobacter pylori	HP1520[5]	KIQKTSLDFSKSNSYLLYAQNGVFKTSFAKSLTD
Yersinia pestis	YFEE[6]	PALLSTLISIINFVYL-YSQKGTGVNAVMLDFIH

Figure 35: Multiple sequence alignment obtained from BLAST expasy search with DFSYLLYSQKG in following databases: Swiss-Prot + TrEMBL + TrEMBL_NEW (complete). *Names of organisms (left), of genes coding for aligned proteins or hypothetical proteins (middle), aligned sequences (right) and protein informations (below) are shown. The similarity search was performed with a threshold value of 100 by using the PAM30 matrix and obtained sequences were filtered for low complexity regions. Gapped alignments were allowed and only sequences with similarity of seven out of eleven amino acid residues present in DFSYLLYSQKG and with two conserved tyrosine residues within the palindrome were accepted. Shown is a gapped, reconstructed alignment of sequences obtained from BLAST, respectively.*

[2,4,5] *Hypothetical proteins*
[3] *Erythrocyte membrane protein PFEMP3*
[6] *Putative yfeABCD regulator yfeE*

V. Discussion

1. Regulated proteolysis of RPTPκ

1.1. Multiple proteases are involved in RPTPκ processing

Some type I membrane proteins have been reported to undergo regulated proteolysis at several sites. More importantly, in some cases such proteolytic events are absolutely required for signal transmission that is mediated by those receptor proteins, as reported for Notch. In the course of this study we described two different cleavage events that produce two κ isoforms, and, taking convertase-catalyzed S1 cleavage into account, there are, altogether, three processing steps that target κ. So, RPTPκ is one of a group of transmembrane receptors that are proteolytically processed in an extremely complex way. For instance, Notch, Cadherins and APP are targeted by at least three cleavage events as well.

In analogy to the notations used to describe the processing of Notch we have designated the individual specific cleavages within the κ sequence as S1, S2 and S3, emphasizing the different cleavage sites, respectively (Fig. 36).

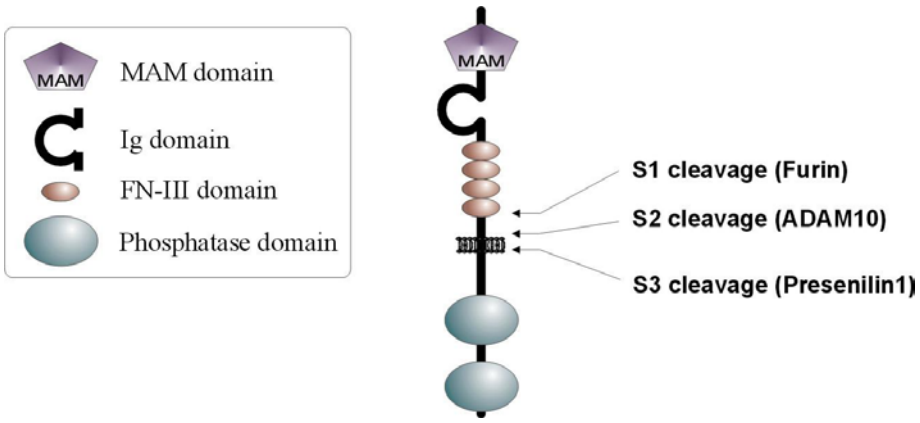

Figure 36: Proteolytic cleavage of RPTPκ. *The cleavage sites are indicated, as are the proteases involved in processing.*

1.2. S1 processing of MAM-family RPTPs

Severeal MAM-family RPTPs have been described, including RPTPκ, μ, ρ, λ and PCP2 (Jiang et al., 1993; Brady-Kalnay et al., 1993; Wang et al., 1996; Cheng et al., 1997; McAndrew et al., 1998). Intriguingly, not all of these RPTPs undergo cleavage at site 1. For

instance, PCP-2 lacks the dibasic furin-like protease recognition motif RxxR within its fibronectin-like domain sequence (Wang et al., 1996).

By using furin knock-out cells we demonstrated the involvement of furin in RPTPκ cleavage at site 1. We also showed that, as a result of furin-mediated cleavage, κ is presented at the cell surface as a protein composed out of two subunits and therefore assigned the secretory pathway as the main compartment responsible for S1 cleavage. In contrast, Campan et al. reported that RPTPμ is processed at S1 by PC5, a protease related to furin and mainly expressed in endothelial cells (Campan et al., 1996). In accordance with this observation, expression of μ was demonstrated in endothelium (Thesis, Miriam Fuchs). However, the authors approach was solely based on protease overexpression experiments that did not interfere with endogene PC5 function.

The functional consequences of S1 processing of RPTPκ are not understood, but it can be envisioned that the two-subunit architecture allows for a more sophisticated regulation of the phosphatase. For example, maturation of Notch by furin-mediated S1 cleavage does also produce a heterodimer, and as a consequence of subunit dissociation induced by calcium chelation the Notch signaling becomes activated (Rand et al., 1999). Thus, the presence of the Notch extracellular subunit generated by S1 processing keeps the protein in an inactive state, so that activation can only be achieved by either dissociation of the E-subunit or additional cleavage that results in shedding of the extracellular fragment.

1.3. S2 processing induced by cell density and phenothiazines

Initially, while studying the MAM family member RPTPλ, Cheng et al. detected the λ-specific (extracellular) E-subunit in cell supernatants and from their studies they concluded that the linkage between the E-and P-subunit was lost. Also, Tonks and colleagues discussed a possible role for S1 processing of RPTPμ in shedding of its extracellular portion (Brady-Kalnay and Tonks, 1995). In contrast, inducible cleavage downstream of S1 and shedding has been reported for PTP-R2A subfamily members, including LAR and RPTPδ (Aicher et al., 1997).

We described processing of the prototypic MAM family member κ “downstream” of the S1 site, i.e. within the extracellular fragment close to the membrane (S2 cleavage). Processing at S2 resulted in shedding. Here we clearly excluded the possibility of subunit dissociation by showing that a furin-cleavage site mutant (that resembles the precursor) is still shed with the result that the size of the released fragment is bigger than that of the E-subunit. Moreover,

processing at S2 was inducible and found to be strongly promoted by phenothiazines, a class of compounds used as neuroleptica. TFP has already been reported as shedding inducer of several cell surface receptors like selectins (Kahn et al., 1998).

One suggested mechanism of TFP action involves the rapid increase of the cytoplasmic calcium concentration that is thought to be the result of interference with calmodulin function in cells or neurons. Additionally, given to the overall hydrophobicity of this compound, insertion of TFP molecules into cell membranes has been demonstrated (Hidalgo et al., 2004). Although S2 cleavage was sensitive to cytoplasmic calcium chelation, it is rather unprobably that κ processing is activated by an increase in cytoplasmic calcium. For instance, the calcium ionophor ionomycin failed to induce remarkable S2 cleavage of κ in most of the cells lines tested. Also, the TFP concentrations used in our assay (100μM) were rather high so that membrane insertion by TFP is a very possible mechanism of action. Moreover, we showed that S2 cleavage strictly depends on the functionality of the endocytic pathway (see below). It has, however, became clear that endocytosis requires cytoplasmic calcium (Abenavoli et al., 2001), indicating that blocking endocytosis by calcium chelation may also diminish processing at S2.

We reported BB-94 as potent inhibitor of S2 cleavage and shedding, pointing to the involvement of metalloproteases. Given the fact that ADAMs are the main mediators of numerous ectodomain shedding events, they are good candidates for mediating S2 processing of κ. Experiments performed by Philipp Mertins (diploma thesis) clearly demonstrated that the function of ADAM10 is required for S2 cleavage of κ. In contrast, processing and shedding of LAR was shown to be induced by PMA and to depend on ADAM17 (Aicher et al., 1997 and doctoral thesis, Jens Ruhe). Thus, the mechanisms leading to processing of κ and LAR are strikingly different with respect to cleavage induction and to the identity of the proteases involved. These contrasting observations are, however, not surprising since both RPTP extracellular fragments are structurally and functionally distinguishable, targeting both RPTPs to different cellular compartments, i.e. MAM family members localize at sites of cell contacts whereas LAR is preferentially localized at focal adhesions.

Density dependent inhibition of growth is one of the most prominent characteristics of normal cells grown in monolayer. This feature is frequently lost in malignantly transformed cells. Little is known about the cellular mechanism underlying this phenomenon. The importance of reversible tyrosine phosphorylation in signaling pathways leading to cell proliferation has,

however, led to the proposal that PTPs may be involved. This is supported by the early finding that treatment with the PTP inhibitor vanadate decreased density dependend growth inhibition (Klarlund, 1985). Membrane fractions derived from dense cells also show an increase in PTP activity compared to those derived from sparse cells (Pallen and Tong, 1991; Gaits et al., 1995). It is therefore not surprising that the expression of several PTPs like DEP-1 (Östman et al., 1994), LMW-PTP (Fiaschi et al., 2001) and the MAM family members κ (Fuchs et al., 1996) and μ (Gebbink et al., 1995) becomes upregulated in cell monolayers. However, it should be realized that upregulation of protein expression is only one mechanism leading to an overall increase in cellular PTP activity. In particular, modulation of enzymatic PTP activity has to be considered.

We identified cell density as one physiological stimulus leading to S2 cleavage. This observation is interesting in regard to the homophilic binding behaviour of MAM family RPTPs. It is therefore conceivable that homophilic binding accomplished between κ proteins expressed in trans triggers their cleavage. Such a mechanism has been reported for trans-interactions involving Notch and its ligand, delta (Mumm et al., 2000), and ephrin-A2 and its receptor, Eph3A (Hattori et al., 2000). For instance, clustered EphA3-Fc chimaeras supplemented in cell supernatants rapidly induced shedding of ephrin-A2 expressed on cells, a mechanism suggested to be mediated by ADAM10. Moreover, interactions between ephrin-A2 and ADAM10 have been shown to depend on a stretch of 10 amino acids located within the ephrin-A2 extracellular sequence.

By deleting the RPTPκ E-subunit, we showed that shedding of the remaining P-stulk was indeed diminished, pointing to the involvement of a mechanism related to the one reported for activation of Notch and ephrin cleavage, i.e. mediation via trans-interactions involving the E-subunits. This mechanistical concept of regulation is also supported by the fact that cell treatment with an antibody recognizing κ's extracellular fragment resulted in induction of rapid processing of endogene expressed κ at S2. Intriguingly, the same antibody was actually shown to decrease ß-catenin tyrosine phosphorylation when added to cells.

Undoubtly, one important question is of whether cleavage at S2 modulates κ's phosphatase activity. Although we observed a strong decrease of ß-catenin tyrosine phosphorylation in response to treatment with the shedding inducer TFP, results obtained from in vitro PTP assays pointed out that (I) TFP did not led to increased κ-specific enzymatic activity and (II) even the uncleaved P-subunit is characterized by high intrinsic phosphatase activity. It thus

seems that cleavage at S2 does not lead to increased κ enzymatic activity in vitro and that TFP-induced dephosphorylation of ß-catenin likely proceeds via mechanisms not involving S2 cleavage of κ. As mentioned above, TFP treatment increases the concentration of cytoplasmic calcium in cells and several PTPs, including PTP1B (Frangioni et al., 1993; Rock et al., 1997), PTP-MEG (Gu and Majerus, 1996) and PTP-STEP (Nguyen et al., 1999; Gurd et al., 1999) have been shown to be cleaved by calpain, a protease activated by calcium. For instance, calpain-catalyzed cleavage results in two-fold activation of PTP1B, implying that ß-catenin dephosphorylation by PTP1B could be accelerated upon TFP stimulation.

The observation of decelerated κ turnover in the presence of BB-94 suggests a role of S2 processing in promoting κ's internalisation (Fig. 37). Indeed, several conditions that blocked receptor internalisation actually diminished S2 cleavage, indicating that particular steps of the endocytic pathway are required for S2 processing. For instance, endocytosis could provide a protein coat or a scaffold, serving to immobilize the protease in close contact with κ or, alternatively, could enforce a conformational change of κ, resulting in exposure of the cleavage site. In this regard, several reports (Selkoe, 1998; Buxbaum et al., 1998; Lammich et al., 1999; Tomita et al., 1998) have shown that ectodomain cleavage of APP by ADAM10 or TACE does occur in early endosomes subsequent to internalization of the protein. Like κ, processing of APP is inhibited by K^+ depletion (Zhou et al., 2000). Another receptor whose cleavage is blocked by K^+ depletion is ErbB4/HER4. ErbB4 was formerly demonstrated to be cleaved by TACE (Rio et al., 2000) in response to heregulin ß1 stimulation (Zhou et al., 2000). So, from these studies it was suggested that endocytosis of the heregulin ß1-ErbB4 complex is necessary for ligand-induced cleavage of ErbB4.

In case of Notch and its ligand Delta, this mechanistical concept has been analysed in detail. Initial studies in Drosophila revealed that activation of Notch requires Dynamin, a GTPase involved in pinching off clathrin-coated vesicles during endocytosis (Seugnet et al., 1997). Parks et al. later demonstrated (also in Drosophila) that endocytosis of Delta into Delta-expressing cells, by forming a tight complex with the Notch extracellular part, drives Notch dissociation, i.e. dissociation of the Notch extracellular and transmembrane subunit in such a way that the Notch extracellular part becomes transendocytosed into Delta-expressing cells (Parks et al., 2000). Moreover, the ubiquitin E3 ligase Neutralized was shown to promote endocytosis of Delta in the signal donor cell and transendocytosis of the Notch extracellular fragment (Pavlopoulos et al., 2001). Although these studies did not discern the

molecular mechanism underlying Notch "dissociation", it was proposed that a mechanical force imparted by endocytosis is linked to a conformational change that exposes the S2 cleavage site (see above). In fact, the functions of both, the S2-cleaving protease ADAM10 and the ubiquitin ligase Neutralized are required for signaling via Notch. Nevertheless, it remains to be addressed how S2 cleavage of Notch is mechanistically linked to endocytosis of Delta and Notch itself.

Internalization of cell surface receptors into endocytic compartments is most often achieved via clathrin- or caveolae-dependent trafficking pathways, both of which can be differentiated by exposure to particular inhibitory conditions, chemical inhibitors or dominant-negative versions of proteins that specifically participate in the internalisation process. For instance, chlorpromazine treatment (Wang et al., 1993) and potassium depletion (Brodsky et al., 2001) were shown to inhibit the clathrin-dependent pathway as does, in a more specific way, expression of dominant-negative Eps15. Intriguingly, our studies revealed

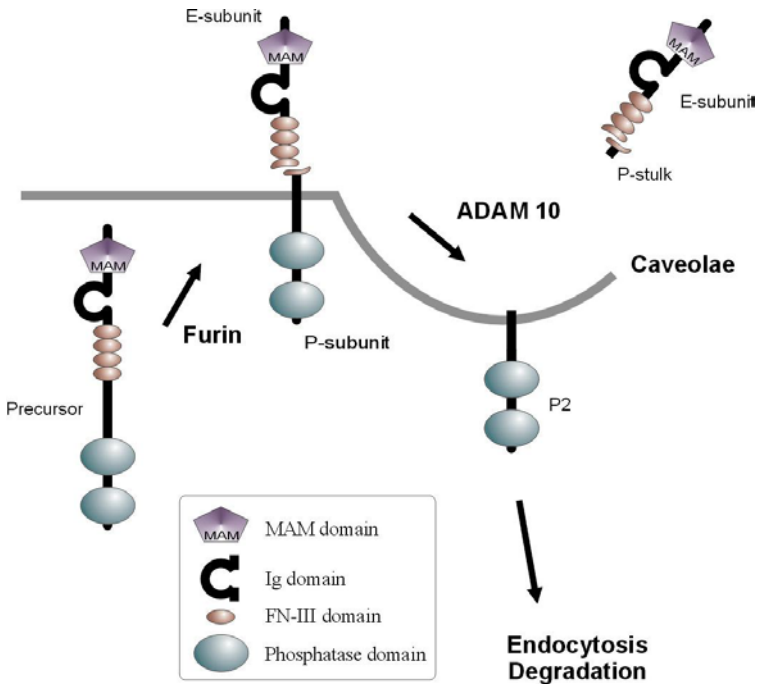

Figure 37: Proposed model for RPTPκ processing. *Furin-mediated cleavage within the membrane-proximal fibronectin-type III domain constitutively yields two-subunit RPTPκ proteins. In contrast, S2 cleavage by ADAM10 is induced at high cell density or upon phenothiazine exposure, resulting in the release of the extracellular portion into the cell culture medium. The model suggests that ADAM10-catalysed cleavage proceeds at initial steps of the endocytic pathway, implicating S2 cleavage in κ internalisation and degradation.*

that chlorpromazine and derivatives thereof actually activated S2 processing of κ, whereas K^+ depletion inhibited it. Importantly, specifically blocking clathrin-mediated endocytosis by

introduction of dominant negative Eps15 did not block cleavage at S2, excluding the involvement of clathrin-dependent endocytosis in κ processing. However, our results do not exclude the contribution of clathrin-dependent internalisation in basal shedding of κ. On the other hand, we demonstrated inhibition of S2 cleavage upon extraction of cholesterol from cell membranes by using the cholesterol binding drug methyl-ß-cyclodextrin, indicating that this cleavage proceeds via caveolae-dependent endocytosis. In general, caveolae perform internalisation more slowly and the resulting vesicles do not become acidified, explaining of why chloroquine and dansylcadaverin failed to diminish κ cleavage at S2. Also, caveolae-mediated internalisation does not constitute a constitutive process (Thomsen et al., 2002) and only occurs upon cell stimulation. Importantly, caveolae have been recognized as major initiating centers for signaling within the cell (Ceresa and Schmid, 2000; Simons and Toomre, 2000).

1.4. Presenilin 1-mediated cleavage at S3 and deregulation in tumorigenesis

Initially, a cytoplasmic κ isoform (P3) was detected in human kidney tissue, and certainly such a membrane-cytoplasmic translocation results in a change of the spectrum of substrates dephosphorylated by P3. Paradoxically, P3 was not found in most of the cell lines analysed by immunoprecipitation procedures from whole cell lysates. By using cells stably expressing presenilin 1 or a dominant negative version thereof, we demonstrated that P3 is the product of presenilin 1-mediated processing (Fig. 38). In fact, inhibition of endogenous Presenilin 1 by introduction of a dominant-negative version led to accumulation of the ADAM product P2, whereas P3 disappeard. This observation is in accord with previous data obtained from studies of Notch signaling, showing that Presenilins only target receptors with a short extracellular stulk region previously generated by ADAM proteases. Presenilins cleave their subtrate receptors exclusively within the hydrophobic membrane environment, i.e. within their transmembrane regions, a mechanism designated as Rip (regulated intramembrane proteolysis).

Interestingly, the intracellular domains of proteins that undergo Rip like Notch, N-Cadherin and Ire1, are transported into the cell nucleus where they modulate the transcription of genes. In this regard, κ's cellular localization has to be analysed. Importantly, the juxtamembrane, ß-catenin-binding sequence is still present in P3, indicating that it probably

retains the ability to bind to ß-catenin, even in the cytoplasm or in the nucleus. Moreover, Presenilins have been shown to associate, like κ, with the ß-catenin-Cadherin complex (Zhou et al., 1997; Murayama et al., 1998; Yu et al., 1998) at sites of cell-cell contact, probably explaining the fact of why P3 preferentially accumulates in compact tissues but not in most of the cells grown in culture. Increasing evidence supports the concept that Preseniln 1 is an important negative regulator of ß-catenin signalling. For instance, Presenilin 1 deficiency in primary fibroblasts leads to stabilization of free ß-catenin (Zhang et al., 1998; Kang et al., 1999; Soriano et al., 2001). In agreement with this finding, loss of Presenilin 1 was associated with elevated Cyclin D1 transcription and accelerated proliferation in cultured cells, as well as epidermal hyperplasias and tumors in vivo in an animal model of Presenilin 1 deficiency (Xia et al., 2001).

The failure of damaged cells to properly respond to previously established mechanical contacts to their neighbor cells can ultimately lead to malignant growth. Albeit the phenomenon of contact inhibition of cell growth is lost in tumors, the molecular mechanism underlying this important growth inhibitory process is still poorly understood. We identified cell density as one stimulus leading to S2 cleavage of κ, indicating that cell density may also cause nuclear accumulation of the κPTP domains by inducing sequential processing at S2 and S3, respectively. Such a signaling pathway would constitute an optimal molecular device to transmit the "cell contact signal" at high cell density from the extracellular environment directly into the nucleus. In fact, given to their homophilic binding behavior, MAM family RPTPs fulfill all criteria needed for sensing cell-cell contacts into the cell interior. Recently, RPTPρ was found to be mutational inactivated in a panel of colorectal cancers, clearly pointing to a tumorsuppressive function (Wang et al., 2004).

The data demonstrated in this thesis indicate that in particular the Presenilin-mediated RPTPκ cleavage product P3 fulfills a putative tumorsuppressive function. We showed that the relative abundance of P3 is decreased in 10 out of 25 primary renal carcinoma samples by more than 90% as compared to surrounding normal tissue, whereas such downregulation in case of the P-subunit was only detected in 3 samples. RPTPκ mRNA expression was found to be decreased in only 1 out of 18 samples tested. It is therefore conceivable that, in tumor tissue, either presenilin-1-mediated processing is specifically inhibited or degradation of P3 is accelerated. Experimental data obtained from Markus Schmid (Diploma thesis) clearly excluded the fact that nucleotide transitions within the κ sequence could account for the

decreases in P3 observed in these renal carcinoma probes. It will therefore be important to determine if the dimished P3 accumulation observed in tumor tissue is due to inactivation of Presenilin itself. Altogether, the RPTPκ Presenilin cleavage product P3 serves a potential tumorsuppressive function and, in this regard, a possible involvement of P3 in nuclear signaling that eventually leads to a proliferation stop has to be addressed in the future.

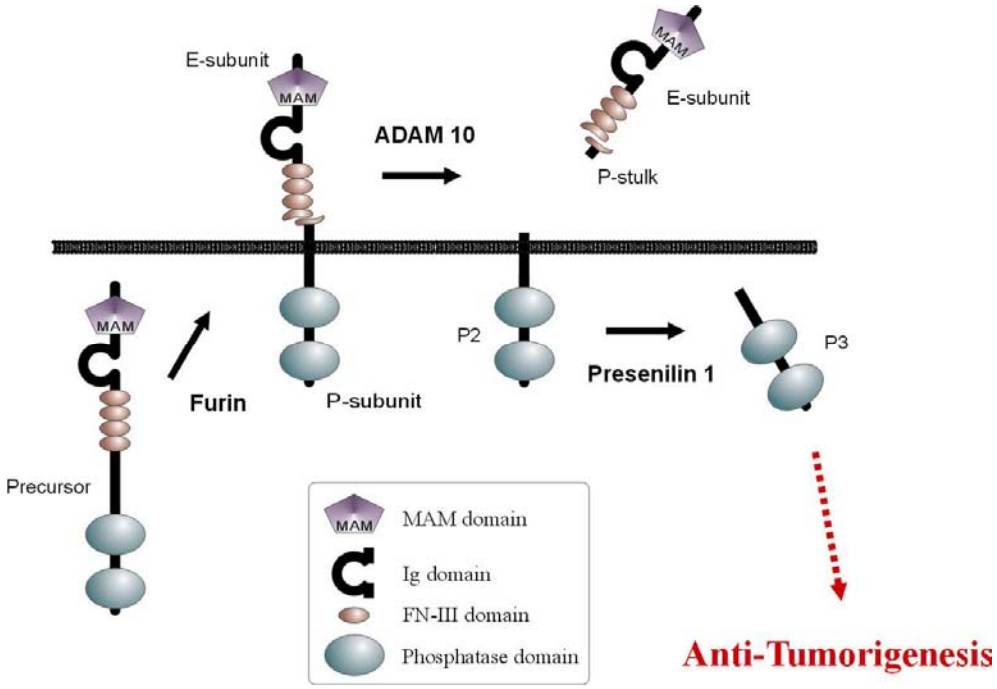

Figure 38: Model summarizing S1, S2 and S3 cleavage of RPTPκ. *Furin-mediated S1 cleavage, ADAM10-catalyzed S2 cleavage and S3 processing, also named Rip (regulated intramembrane proteolysis), mediated by Presenilin 1. The latter results in "solubilization" of both PTP domains. Importantly, P3 accumulation is decreased by more than 90% in 10 out of 25 primary human kidney tumors, indicating that P3 serves an anti-tumorigenic function.*

2. Involvement of the κ MAM-derived palindromic sequence in binding to Plasminogen

A plethora of ligands have been described for RTKs, ranging from small, soluble polypeptides like EGF to type I transmembrane proteins including the ephrins. Certain RTKs like Axl have the ability to undergo homophilic interactions. In many cases, one RTK binds to several different ligands. For RPTPs, the search for such ligands is one area in which further progress needs to be made. Although MAM family RPTPs are characterized by the ability of homophilic binding, we searched for potential soluble ligands by using GST pulldown assays. This approach led to the identification of Plasminogen as κ MAM domain binding protein.

Plasminogen is synthesized in the liver and maintained in plasma at a stable concentration of approximately 200µg/ml. It is the inactive precursor of Plasmin, a potent serine protease involved in destruction of fibrin and other extracellular matrix proteins (Andreasen et al., 1997). Plasmin also catalyses activation of latent transforming growth factor ß and release of basic fibroblast growth factor from its extracellular binding sites (Seghezzi et al., 2002) and has been implicated in shedding of cell adhesion molecules like NCAM and E-Cadherin (Nayeem et al., 1999; Endo et al., 1998; Endo et al., 1999; Ryniers et al., 2002). Gene disruption experiments confirmed physiological importance of Plasmin in activation of MMP-2, MMP-3, MMP-9 and MMP-13 (Carmeliet et al., 1997; Lijnen et al., 1998a; Lijnen et al., 1998b). In light of these reports it is not surprising that Plasminogen ablation in mice results in reduction of tumor growth and metastasis formation (Bugge et al., 1997; Bugge et al., 1998).

Conversion of Plasminogen to Plasmin is performed by its natural activators, t-PA or u-PA, or by bacterial products like streptokinase. Importantly, such Plasminogen activators are used clinically as clot-dissolving (thrombolytic) agents for treatment of pulmonary embolism and acute myocardial infarction. Several reports have demonstrated lysine-dependent binding of Plasminogen to the surface of cancer cells where it colocalizes with u-PA. Accordingly, patients with higher tumor u-PA levels have a shorter disease-free interval and a shorter overall survival than patients with a lower (Andreasen et al., 1997; Schmitt et al., 1997). Moreover, u-PA ablation in mice results in drastically reduced progression to chemically induced malignant melanomas (Shapiro et al., 1996). Taken together, the Plasminogen

activation system plays an important, multifunctional role in tumor biology. During the last decade it became clear that, in analogy to metastatic tumor cells, bacterial pathogens make use of the Plasminogen/Plasmin reception in order to penetrate through tissue barriers like basement membranes and the extracellular matrix. For instance, by using the Plg-/- mice, Coleman et al. demonstrated a role for Plasminogen in the establishment of the *Borrelia burgdorferi* bacteremia. Table 6 summarizes several reports describing bacterial Plasminogen/Plasmin receptors.

Binding studies with κMAM-derived peptide libraries demonstrated that Plasminogen targets the palindromic core sequence DFSYLLYSQK, which is not present in the MAM domain of RPTPμ, respectively. By substitutional analysis we showed that the residues D, Y, Y and K are involved in Plasminogen binding. The fact that the motif contains a palindromic core sequence is interesting, however, the importance of palindromes in mediating protein-protein interactions in general is unclear at present. The observation that binding is restored upon D to E and K to R substitutions demonstrates the importance of the charged amino acid side chains in binding. From our data we concluded a model in which the two side chains of the negative charged D and the positive charged K form an ion bridge (molecular clamp), thereby exposing the hydrophobic loop structure that contains the two tyrosine residues which are recognized by Plasminogen.

The fact that proteins from pathogenic bacteria show sequence similarity to the identified binding motif is interesting. However, none of these proteins have been implicated in Plasminogen binding to date. PfEMP3 and yfeE have been characterized regarding to their function. Upon red blood cell infection by the murine malaria parasite *Plasmodium yoelii yoelii*, Plasmodium encoded PfEMP3 is exported to the cell membrane of the infected host cell where it interacts with the membrane skeleton, thereby modulating membrane regidification (Glenister et al., 2002). YfeE was recognized as virulence factor for *Yersinia pestis*, the causative agent of bubonic plague. It is a component of an ABC transporter system (ATP-binding cassette) involved in the acquisition of iron. Note that both proteins are localized at the cell surface. In this regard it should be emphasized that most, if not all, Plasminogen-binding proteins are directly or indirectly involved in conversion of Plasminogen to Plasmin, thereby contributing to the proteolysis of extracellular proteins or protein fragments (Table 6).

Microbial species	Original references	Further references
Staphylococcus aureus	Kuusela P, Saksela O.: Binding and activation of plasminogen at the surface of Staphylococcus aureus. Increase in affinity after conversion to the Lys form of the ligand.	
Streptococcus pyogenes	Lottenberg,R.; Broder,C.C.; Boyle,M.D.: Identification of a specific receptor for plasmin on a group A streptococcus. Ullberg, M., Kronvall, G., and Wiman, B.: New receptor for human plasminogen on gram positive cocci Lottenberg, R., Broder, C.C., Boyle, M.D., Kain, S.J., Schroeder, BL, and Curtiss, R.: Cloning, sequence analysis, and expression in Escherichia coli of a streptococcal plasmin receptor.	Poon-King, R., Bannan, J., Viteri, A., Cu, G., and Zabriskie, J.B.: Identification of an extracellular plasmin binding protein from nephritogenic streptococci. Berge, A. and Sjöbring, U.: PAM, a novel plasminogen-binding protein from Streptococcus pyogenes.
Streptococci group C, G	Ullberg, M., Kronvall, G., and Wiman, B.: New receptor for human plasminogen on gram positive cocci.	Ullberg, M., Karlsson, I., Wiman, B., and Kronvall, G.: Two types of receptors for human plasminogen on group G streptococci.(1992)
Streptococcus equisimilis	Gase, K., Gase, A., Schirmer, H., and Malke, H.: Cloning, sequencing and functional overexpression of the Streptococcus equisimilis H46A gapC gene encoding a glyceraldehyde-3-phosphate dehydrogenase that also functions as a plasmin(ogen)-binding protein. Purification and biochemical characterization of the protein.	
Streptococcus pneumoniae	Ullberg, M., Kronvall, G., and Wiman, B.: New receptor for human plasminogen on gram positive cocci.	
Haemophilus influenzae	Ullberg, M., Kronvall, G., Karlsson, I., and Wiman, B.: Receptors for human plasminogen on gram-negative bacteria.(1990)	Sjöström, I., Gröndahl, H., Falk, G., Kronvall, G., and Ullberg, M.: Purification and characterisation of a plasminogen-binding protein from Haemophilus influenzae. Sequence determination reveals identity with aspartase. (1997).
Moraxella catarrhalis	Ullberg, M., Kronvall, G., Karlsson, I., and Wiman, B.: Receptors for human plasminogen on gram-negative bacteria. (1990).	
Escherichia coli	Korhonen, T.K., Lähteenmäki, K., Kukkonen, M., Pouttu, R., Hynonen, U., Savolainen, K., Westerlund-Wikström, B., and Virkola, R.: Plasminogen receptors. Turning Salmonella and Escherichia coli into proteolytic organisms.	
Proteus mirabilis	Ullberg, M., Kronvall, G., Karlsson, I., and Wiman, B.: Receptors for human plasminogen on gram-negative bacteria. (1990)	
Pseudomonas aeeruginosa	Ullberg, M., Kronvall, G., Karlsson, I., and Wiman, B.: Receptors for human plasminogen on gram-negative bacteria. (1990).	
N. gonorrhoeae, N. meningit	Ullberg, M., Kuusela, P., Kristiansen, B.E., and Kronvall, G.: Binding of Plasminogen to Neisseria meningitidis and Neisseria gonorrhoeae and Formation of Surface-Associated Plasmin.	
Borrelia burgdorferi	Fuchs, H., Wallich, R., Simon, M.M., and Kramer, M.D.: The outer surface protein A of the spirochete Borrelia burgdorferi is a plasmin(ogen) receptor. Klempner, M.S., Noring, R., Epstein, M.P., McCloud, B., Hu, R., Limentani, S.A., and Rogers, R.A.: Binding of human plasminogen and urokinase-type plasminogen activator to the Lyme disease spirochete, Borrelia burgdorferi.	Hu, L.T., Pratt, S.D., Perides, G., Katz, L., Rogers, R.A., and Klempner, M.S.: Isolation, cloning, and expression of a 70-kilodalton plasminogen binding protein of Borrelia burgdorferi.

Table 6: Investigations demonstrating reception of Plasminogen/Plasmin on the surface of pathogenic bacteria.

VI. Summary

In this thesis it is demonstrated that furin is required for constitutive processing of RPTPκ within the secretory pathway. We next showed that κ is specifically targeted by two additional cleavages that we named S2 and S3 to specify the result of processing at sites 2 and 3, sites downstream of S1. Processing at S2 is mediated by metalloproteases and results in RPTPκ shedding. It was found to be promoted by cell density and phenothiazine exposure and κ's extracellular subunit was required for shedding induced by both stimuli. Moreover, conditions that blocked the functionality of caveolae-dependent receptor internalisation into endocytic compartments diminished S2 cleavage, indicating that it proceeds in the course of internalisation via caveolae. In addition, Presenilin 1 was shown to further cleave P2, the product of S2 processing, thereby generating the cytoplasmic κ isoform P3, accumulation of which is diminished by more than 90% in 10 out of 25 primary human kidney tumors.

Plasminogen was identified as RPTPκMAM domain binding protein. Binding studies performed with κMAM derived peptide libraries demonstrated that Plasminogen targets the palindromic sequence DFSYLLYSQK and substitutional analysis revealed that the residues D, Y, Y and K are involved in binding. Similar motifes were identified in proteins derived from pathogenic bacteria.

VI. Zusammenfassung

In der vorliegenden Arbeit wurde gezeigt, dass die konstitutive proteolytische Prozessierung der RPTPκ durch die Protein Convertase Furin erfolgt. Es wurden weitere, spezifische Prozessierungen der RPTPκ identifiziert, die als S2- und S3- Spaltungen bezeichnet wurden. Die S2-Prozessierung erfolgt durch Metalloproteasen und resultiert in der Freisetzung des extrazellulären Fragments in das Zellmedium. Als Stimuli dieser Prozessierung konnten hohe Zelldichten und Phenothiazine identifiziert werden. Für die Induktion der S2-Spaltung durch beide Stimuli ist das Vorhandensein des RPTPκ extrazellulären Fragments notwendig. Ein zweiter, notwendinger Faktor ist die Funktionalität von Endozytose-Prozessen die von Caveolae (Cholesterol-reiche Membranareale) abhängig sind. Das S2-Spaltprodukt P2 wird durch Presenilin-1 weiter prozessiert (S3), was schliesslich zur Freisetzung der cytoplasmatischen κ-Isoform P3 führt, deren Akkumulierung in 10 von 25 primären, humanen Nierentumoren um mehr als 90% verringert ist.

Plasminogen wurde als ein mit der MAM-Domäne der RPTPκ interagierendes Protein identifiziert. Plasminogen bindet an die palindromische Sequenz DFSYLLYSQK und die Aminosäurereste D, Y, Y und K sind direkt in die Interaktion involviert. Ähnliche Sequenzmotive konnten in Proteinen von pathogenen Bakterien identifiziert werden.

VII. References

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VIII. Appendix

Abbreviations

ADAM	A disintegrin and metalloprotease domain
Amp^r	Ampicilline resistence
APC	Adenomatous polyposis coli gene
ATP	Adenosintriphosphate
bp	Base pairs
BSA	Bovine serum albumin
°C	Degree celsius
Ca^{2+}	Calcium Ions
CD45	Cluster of Differentiation 45
cDNA	Complementary DNA
DEP-1	Density-enhanced phosphatase 1
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Desoxyribonukleic acid
dsDNA	Dooble-stranded DNA
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethlendiamintetraacetate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
GST	Glutathion-S-transferase
GTP	Guanosintriphosphate
h	Hour
HA	Hemagglutinin
HEPES	N-(2-Hydroxyethyl)-piperazin-N‘-2-Ethansulfonic acid
IP	Immunoprecipitation
kb	Kilobase
kDa	Kilodalton
µ	Micro
LAR	Leucocyte antigen-related phosphatase
LMW-PTP	Low molecular weight PTP
m	Milli
M	Molar
MAM	Meprin, A5 glycoprotein, RPTPm
MAP	Mitogen-activated protein
MAPK	MAP kinase
min	Minute
MMP	Matrix metalloprotease
NCAM	Neural cell adhesion molecule
PAGE	Polyacrylamide gel elektrophoresis

PBS	Phosphate-buffered saline
PC	Protein convertase
PCP-2	Pancreatic carcinoma-derived phosphatase 2
PCR	Polymerase chain reaction
PEG	Polyethylenglycole
PDGFR	Plateled-derived growth factor receptor
PMA	12-O-Tetradecanoyl-phorbol-13-acetat
pNPP	p-Nitrophenyl-phosphate
pRS	pRetroSUPER vector
PS1	Presenilin 1
PTP	Phosphotyrosine-specific phosphatase
PTP-MEG	Megakariocyte PTP
PTP-Pez	Band 4.1/ezrin-related protein tyrosine phosphatase
pY	Phosphotyrosine
RPTP	Receptor-like Phosphotyrosine-specific phosphatase
rpm	Rotations per minute
RT	Room temperature
RTK	Receptor tyrosine kinase
SDS	Natriumdodecylsulfate
SH	Src homology
SHP-1	SH2-containing PTP-1
SHP-2	SH2-containing PTP-2
Src	Homologue to v-src (sarcoma viral oncogene)
TC-PTP	T-cell phosphatase
Tris	Tris(hydroxymethyl)aminomethan
o.N.	Overnight
UV	Ultraviolett
V	Volt
VSV	Vesicular stomatitis virus glycoprotein VSV-G
Vol	Volume
WB	Western Blot
WT	Wild type

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