The PTPµ protein tyrosine phosphatase binds and recruits the scaffolding protein RACK1 to cell-cell contacts

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Running title: PTPµ recruits RACK1 to points of cell contact
PTPμ, an Ig-superfamily receptor protein tyrosine phosphatase (RPTP), promotes cell-cell adhesion and interacts with the cadherin/catenin complex. The signaling pathway downstream of PTPμ is unknown; therefore, we used a yeast two-hybrid screen to identify additional PTPμ interacting proteins. The membrane-proximal catalytic domain of PTPμ was used as bait. Sequencing of two positive clones identified the scaffolding protein RACK1 (receptor for activated protein C kinase) as a PTPμ interacting protein. We demonstrate that RACK1 interacts with PTPμ when co-expressed in a recombinant baculovirus expression system. RACK1 is known to bind to the src protein tyrosine kinase (PTK). This study demonstrates that PTPμ association with RACK1 is disrupted by the presence of constitutively active src. RACK1 is thought to be a scaffolding protein that recruits proteins to the plasma membrane via an unknown mechanism. We have shown that the association of endogenous PTPμ and RACK1 in a lung cell line is increased at high cell density. We also demonstrate that the recruitment of RACK1 to both the plasma membrane and cell-cell contact sites is dependent upon the presence of the PTPμ protein in these cells. Therefore, PTPμ may be one of the proteins that recruits RACK1 to points of cell-cell contact which may be important for PTPμ-dependent signaling in response to cell-cell adhesion.
Introduction

Control of tyrosine phosphorylation is regulated by the opposing actions of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). The PTP superfamily is a diverse group of proteins, which include transmembrane receptors (1). Many of these receptor protein tyrosine phosphatases (RPTPs) are members of the immunoglobulin (Ig) superfamily, a group of proteins responsible for cell recognition or adhesion. We previously demonstrated that the RPTP, PTPµ, promotes cell-cell aggregation when expressed in nonadhesive cells (2-5). These studies demonstrated that the binding is homophilic (i.e., the “ligand” for PTPµ is an identical PTPµ molecule on an adjacent cell). Interestingly, endogenous levels of the PTPµ adhesion molecule have also been shown to promote neurite outgrowth from retinal neurons (6). RPTPs have cell adhesion molecule-like extracellular segments as well as intracellular domains possessing tyrosine phosphatase activity, suggesting they may play a regulatory role in cell adhesion-induced signaling (1,7,8). However, the precise signaling pathways utilized by RPTPs are unknown.

The juxtamembrane domain of PTPµ contains a region of homology to the conserved intracellular domain of the cadherins (9). Cadherins are calcium-dependent cell-cell adhesion molecules that interact with molecules termed catenins that associate with actin (10,11). We previously demonstrated that PTPµ associates with a complex containing classical cadherins, α catenin and β catenin (12-14). In addition, we have recently demonstrated that PTPµ is required for N-cadherin-dependent neurite outgrowth (6). The signal transduction pathways downstream of the RPTPs and cadherins are not well understood. In this manuscript, we demonstrate that
PTPµ interacts with RACK1 and that this protein may be a component of the PTPµ signaling pathway.

A recently identified group of cytosolic proteins called RACKs (receptors for activated protein C kinase) have been shown to bind to PKC only when it is in the activated state (15). It has been suggested that the binding of activated PKC to RACK(s) is necessary for the translocation of PKC to the plasma membrane, a process thought to be required in order for PKC to perform its physiological function (15). A specific RACK, RACK1, has been cloned and is a homolog of the β subunit of heterotrimeric G proteins as determined by the existence of WD repeats (16). WD repeats are 40 amino acid motifs proposed to mediate protein-protein interactions (17). RACK1 is composed of seven WD repeats that are thought to form propeller-like structures (18).

More recent data suggests that RACK1 is a scaffolding protein that recruits a number of signaling molecules into a complex. Theoretically, the seven propellers of the RACK1 structure (18) could bind seven different proteins. RACK1 has been shown to bind PKC, PLCγ, the src cytoplasmic protein tyrosine kinase (PTK), cAMP phosphodiesterase-4, the β subunit of integrins, and the β chain of IL-5R (an interleukin receptor) (19-23). RACK1 has also been demonstrated to bind select pleckstrin homology (PH) domains in vitro including dynamin, β-spectrin, Ras GRF and oxysterol binding protein (OSBP) (24). Some of the interactions between RACK1 and the proteins listed above have been shown to be mutually exclusive (20). In addition, only a subset of these interactions depend upon PKC stimulation (22). These studies
suggest that RACK1 may form distinct signaling complexes in response to unique cellular stimuli.

In this study, we utilized the yeast two-hybrid genetic screen to isolate PTPμ interacting proteins and identified RACK1 as a protein that binds directly to the membrane-proximal catalytic domain in the cytoplasmic segment of PTPμ. We characterized this interaction using a recombinant baculovirus expression system and showed that RACK1 and PTPμ interact only when co-expressed. We also demonstrated that the presence of constitutively active src disrupts the interaction between PTPμ and RACK1. In MvLu cells, which endogenously express both PTPμ and RACK1, we demonstrated that PTPμ and RACK1 associate predominantly at higher cell densities. The association between RACK1 and PTPμ is not affected by activation of PKCs via phorbol esters. PTPμ is upregulated at high cell density in MvLu cells (25) and is primarily found at cell-cell contact sites (12). We have found that RACK1 is recruited to the plasma membrane and points of cell-cell contact at high cell density. Antisense downregulation of PTPμ expression results in a cytoplasmic localization of RACK1 even in the presence of cell contacts. Therefore, the recruitment of RACK1 to both the plasma membrane and cell-cell contact sites is dependent upon PTPμ. Localization of RACK1 to points of cell-cell contact may be an important part of the PTPμ-dependent signal transduction process in response to adhesion.

**Experimental Procedures**

*Yeast Two-hybrid Screen:* We used the LexA version of the yeast two-hybrid system to perform an interaction trap assay (26). This approach detects protein-protein interactions between a
protein from a HeLa cell (human) cDNA library and a construct containing the membrane-proximal catalytic domain of human PTPµ (PTPµD1) as the bait. Amino acids 915-1178 (PTPµ-D1) were cloned in frame with the LexA coding sequence of pEG202 (HIS3) to generate a “bait” plasmid. The resulting construct (pEG202-D1) was sequenced for insertion and correct orientation. The pEG202-D1 plasmid and the betagalactosidase reporter plasmid (pSH18-34) were co-transformed into the yeast strain YPH499. The pSH18-34 (URA3) reporter plasmid contains a LexA-operator-lacZ fusion gene. The pEG202-D1 plasmid did not activate the betagalactosidase reporter plasmid on its own. A HeLa cell human cDNA library (26) in the pJG4-5 (TRP1) yeast expression vector was introduced into a yeast strain containing a chromosomal copy of the LEU2 gene (EGY48) where the activating sequences of the LEU2 gene are replaced with LexA operator sequences. The two strains (EGY48 and YPH499) were mated and the resulting colonies containing the three plasmids were processed according to published methods (26). Potential interactions were detected by growing the mated yeast strain on minimal medium containing 2% galactose and 1% raffinose and lacking the appropriate amino acids to ensure selective pressure of the auxotrophic markers (only colonies containing all the plasmids and expressing the leucine reporter gene will grow). We screened 2.4x10^7 colonies and found four strong interactors. We isolated the prey-containing plasmids and sequenced the DNA from these clones. Two independent positive “prey” clones (clones 1 and 2) were identified as full-length RACK1 by DNA sequencing of the library plasmid (pJG4-5). The positive control used in Figure 1a is a yeast strain containing a self-activating bait plasmid that grows on both glucose and galactose. The negative control for Figure 1b is a yeast strain containing the bait (pEG 202-D1), reporter plasmid (pSH 18-34) and an empty prey vector (pJG4-5). The positive control for
Figure 1b is Etk and HSP70 that interact with one another (H.J. Kung and Yun Qiu personal communication).

A constitutively-active form of the src protein tyrosine kinase (Y^{527}F, Y^{516}F double mutant) was obtained from Dr. Jonathan Cooper (27). The BTM116 plasmid containing the src gene was restriction digested with BamH1. A partial digest of pSH18-34 was performed with BamH1 and the src insert was ligated with this vector. The pSH18-34/src and the pEG202-D1 plasmids were used to transform the YPH499 yeast strain. Then this YPH499 strain was mated to the EGY48 strain containing the RACK1 gene. This allowed us to test whether src interaction with RACK1 could disrupt the RACK1/PTPµ interaction that drove β-galactosidase transcription.

Antibodies: Monoclonal antibodies against the intracellular (SK7, SK15, SK18) and extracellular (BK2) domains of PTPµ or polyclonal antibody against the intracellular domain (471) of PTPµ have been described (2,5). A control monoclonal antibody directed against L1 (8D9) was generously provided by Dr. Vance Lemmon (Case Western Reserve University, Cleveland, OH). A monoclonal antibody to the HA tag conjugated to HRP (Roche Molecular Biochemicals, Indianapolis, IN) was used to detect recombinant RACK1. The HA antibody was also purchased in a biotinylated form and strepavidin-HRP was used for visualization (Covance, Denver, PA). In addition, a specific antibody to RACK1 was purchased from Transduction Labs (Lexington, KY). The monoclonal antibody to src was purchased from CalBiochem (San Diego, CA).
**Baculoviruses:** The baculovirus encoding the intracellular domain of PTPµ (intra-PTPµ) has been described (2). The src baculovirus, which encodes a constitutively active kinase (Y\(^{527}\) F mutant), was kindly provided by Dr. Michael Weber and originally constructed by Dr. David Morgan (28). The RACK1-pJG4-5 vector was restriction digested so that the resulting 1800 bp fragment contained an HA tag (from pJG4-5) in frame with the full-length RACK1 clone. The 1800 bp fragment was cloned into the pAcHLT-C (BD Pharmingen, San Diego, CA) baculovirus expression vector. The construct was sequenced to confirm orientation and correct insertion of the 1800 bp fragment. This created a form of RACK1 that contained a poly-histidine tag, a PKA site, a thrombin cleavage site and an HA-tag, at the N-terminus followed by the RACK1 cDNA sequence (amino acids 1-317). Baculoviruses were generated using the BaculoGold ™ system (BD PharMingen).

**Expression in Insect Cells:** Sf9 cells (CRL 1711; American Type Culture Collection, Rockville, MD), derived from the ovary of the Fall armyworm *Spodoptera frugiperda*, were maintained at 27°C in Grace’s Insect Medium Supplemented (GIBCO-BRL, Grand Island, NY) containing 10% fetal bovine serum and 10 µg/mL gentamicin. The viral stocks were then used to infect Sf9 cells and express the proteins of interest (PTPµ, RACK1 and src). Forty-eight hours post-infection, cells were either lysed or treated with 160nM PMA (activator of PKC-Calbiochem, San Diego, CA) (22) or PP2 (inhibitor of src family kinases-Calbiochem) for 30 min. at 27°C. Cells were lysed in ice-cold buffer (1% Triton X-100, 20 mM Tris pH 7.6, 1 mM benazamidine, 2µl/ml protease inhibitor cocktail (Sigma, St. Louis, MO), 150 mM NaCl, 0.2µM okadaic acid, 200 µM phenylarsine oxide, 1mM vanadate, 0.1mM molybdate) for 30 minutes on ice. The lysates were centrifuged at 3,000g for 3 min. to remove Triton insoluble components.
MvLu Cell Experiments: Mv 1 Lu (MvLu) mink lung epithelial cells (ATCC number CCL 64) were grown at 37°C, 5% CO₂ in DMEM containing 10 μg/mL gentamicin plus 10% fetal bovine serum (GIBCO-BRL). The MvLu cells were grown to 50% or 90% confluence prior to lysis. PKC activation of MvLu cells was performed by adding 10nM PMA (Calbiochem) for 15 min. at 37°C before lysis of the cells. MvLu cells were lysed in buffer (20mM Tris pH 7.6, 1% Triton X-100, 2µl/ml protease inhibitor cocktail, 1mM benzamidine, 200 µM phenyl arsine oxide, 1 mM vanadate and 0.1mM molybdate), incubated on ice for 30 min. and centrifuged at 14,000 x g for 3 min.

A replication-defective amphotrophic retrovirus expressing an antisense PTPμ construct and control retrovirus have been described previously (6). MvLu cells were incubated in virus-containing medium supplemented with serum (10% final) plus 5µg/ml polybrene for 4 days. Reduction in endogenous PTPμ expression was verified by immunoblotting lysates from infected cells with antibodies to PTPμ.

Immunoprecipitations and Electrophoresis: For immunoprecipitation, antibodies to PTPμ or RACK1 were incubated with Protein A sepharose (Pharmacia Biotech, Piscataway, NJ) or Goat anti-mouse IgG (or IgM) that had been conjugated to sepharose (Zymed, South San Francisco, CA) for 2 hours at room temperature then washed 3 times with PBS (phosphate buffered saline: 9.5mM phosphate, 137mM NaCl, pH 7.5) before addition to cell lysates. Purified monoclonal antibodies were used at 0.6mg of IgG/ml beads, ascites fluid was used at 1mg of IgG/ml beads and polyclonal serum was used at 3mg of IgG/ml beads. Immunoprecipitates were prepared
from 40µg (Sf9 cells) or 300µg (MvLu cells) of a Triton-soluble lysate of cells as measured by the Bradford method. The immunoprecipitates were incubated overnight at 4°C on a rocker, centrifuged at 3,000 x g for 1 min. The supernatant was removed from the beads, the beads were washed four times in lysis buffer and the bound material eluted by addition of 100µl of 2X sample buffer and heated for 5 min. at 95°C. One-fifth of the immunoprecipitate (20 µL) was loaded per lane of the gel, the proteins were separated by 10% (for analysis of RACK1 and src), 7.5% (for analysis of the intracellular PTPµ) and 6% (for analysis of endogenous PTPµ) SDS-PAGE and transferred to nitrocellulose for immunoblotting as previously described (2).

**Immunocytochemistry:** All chemicals were diluted in PBS. Cells were fixed with 2% paraformaldehyde for 30 min. at room temperature (Electron Microscopy Sciences, Fort Washington, PA). The cells were permeabilized with 0.5% saponin, blocked with 20% normal goat serum plus 1% BSA and incubated with primary antibody for 18 hours at 4°C. The cells were washed with TNT buffer (0.1M Tris, 0.15M NaCl, 0.05% Tween-20) containing 0.5% saponin and incubated with Texas-Red, Fluorescein or Rhodamine conjugated secondary antibody (Molecular Probes, Eugene, OR or Cappel Research Products, Durham, NC) for 1.5 hours at room temperature. Samples were mounted with Slow fade Light (Molecular Probes, Eugene, OR). The fluorescent labeling was examined using a 40X objective on a Zeiss Axioplan 2 microscope equipped for epifluorescence. Images were captured using a Hamamatsu cooled CCD camera.

**Results**
Yeast two-hybrid analysis

The yeast two-hybrid interaction trap assay (26) was used to identify proteins that were capable of binding directly to the membrane-proximal catalytic domain of PTPµ. Potential interactors were detected by growing the mated yeast strain on minimal medium containing 2% galactose and 1% raffinose and lacking the appropriate amino acids (only colonies containing all the plasmids and expressing the leucine reporter gene will grow). We screened 2.4x10^7 colonies. PTPµ interactors were selected by three criteria. First, they were screened for viability on medium lacking leucine. Only interacting clones will be able to grow on medium without leucine. Second, they were screened for formation of blue colonies when grown on medium containing Xgal/galactose compared to Xgal/glucose containing medium. Galactose specifically induces expression of the “prey” protein whereas glucose does not. Third, they were discriminated for the level of transcriptional activation of the lacZ gene based on the blue color of the colonies when grown on medium containing Xgal. Figure 1 illustrates two independent clones (clones 1 and 2) that fulfilled these criteria. The two clones did not grow on glucose but did grow on galactose (Fig. 1 a). These two clones also expressed high levels of β–galactosidase (Fig. 1 b R&µ). Sequence analysis of these two independent positive clones demonstrated that they both encoded RACK1, a member of the heterotrimeric Gβ superfamily of proteins.

RACK1 and PTPµ interact in Sf9 cells

To characterize the interaction of PTPµ and RACK1, Sf9 cells were infected with baculoviruses encoding full-length RACK1 or the intracellular domain of PTPµ, singly or in
combination. Lysates from Sf9 cells were immunoprecipitated with RACK1 or PTPµ (471) antibodies and resolved by SDS-PAGE. Immunoblots of immunoprecipitates probed with an antibody to RACK1 are shown (Fig. 2 a and b). Immunoblots of PTPµ immunoprecipitates probed with an anti-PTPµ (SK15) antibody are shown (Fig. 2 c). Equal amounts of RACK1 were available in the Triton soluble lysate used for immunoprecipitation based on the ability of RACK1 antibodies to immunoprecipitate RACK1 (Fig. 2 a, lanes 1, 3-4). Figure 2 also illustrates that equal amounts of PTPµ were immunoprecipitated from the PTPµ-infected cell samples (Fig. 2 c, lanes 2-4). PTPµ immunoprecipitates (471 antibody) were immunoblotted with antibody to RACK1 (Fig. 2 b). RACK1 interaction with PTPµ was only detected in cells where both proteins were co-expressed (Fig. 2 b, lane 3-4). The PTPµ antibody did not immunoprecipitate RACK1 from cell lysates in the absence of PTPµ expression (Fig. 2 b, lane 1), thus ruling out the possibility that the antibody recognized RACK1 nonspecifically. Treatment with phorbol esters can stimulate PKC because of their close chemical similarity to diacylglycerol (15). An endogenous PKC that resembles the non-conventional PKCs is expressed in Sf9 cells and is known to be stimulated 20 fold by phorbol ester treatment (29). The interaction between RACK1 and PTPµ was unaffected by endogenous PKC stimulation with phorbol esters (Fig. 2 b, lane 4). These data indicate that RACK1 only bound to PTPµ when both proteins were co-expressed in insect cells and the interaction was not affected by phorbol esters.

**Src disrupts the interaction between RACK1 and PTPµ**

Since RACK1 is known to bind to src (20), we tested whether addition of a constitutively active src to the yeast cells affected binding between RACK1 and PTPµ.
β-galactosidase staining of yeast expressing RACK1 and PTPµ was positive while RACK1/PTPµ/src containing yeast did not turn blue on medium containing Xgal (Fig. 1 b). Therefore, constitutively active src appears to disrupt the interaction between RACK1 and PTPµ in yeast.

We then analyzed whether constitutively active src could disrupt the interaction between RACK1 and PTPµ in the Sf9 cell system. We did single, double or triple infections with RACK1, PTPµ and constitutively active src. Figure 3 shows that PTPµ (panel c) and the src PTK (panel d) were expressed in the appropriate samples. RACK1 antibody immunoprecipitated RACK1 from all the appropriate samples (Fig. 3 a, lanes 1-4). PTPµ immunoprecipitated PTPµ in all the appropriate samples (Fig. 3 c). While RACK1 and PTPµ interact when co-infected (Fig. 3 b, lane 2), there was no interaction detected when src was added in the triple infection (Fig. 3 b, lane 3-4). PP2, the cell permeable src family kinase inhibitor, had no effect on the ability of src to disrupt the PTPµ/RACK1 interaction (Fig. 3 b, lane 4). PP2 was able to inhibit src tyrosine kinase activity as evidenced by a decrease in anti-phosphotyrosine reactivity (Fig. 3 e). These results suggest that the ability of src to disrupt the PTPµ/RACK1 interaction was not dependent upon kinase activity or mediated by tyrosine phosphorylation of any of the proteins. Therefore, constitutively active src was able to disrupt the interaction between RACK1 and PTPµ in yeast as well as in the Sf9 cell system. Together, these results suggest that PTPµ and src may form mutually exclusive complexes with RACK1.

*Endogenous RACK1 interacts with endogenous PTPµ*
To examine whether endogenous PTPµ associates with endogenous RACK1, immunoprecipitation experiments were performed using MvLu cells. Endogenous PTPµ in MvLu cells is proteolytically processed. The full-length form is cleaved into two noncovalently associated fragments, one (P-subunit) comprising the entire intracellular and transmembrane segments and a short stretch of extracellular sequence, the other (E-subunit) containing the remainder of the extracellular segment (5,25). Both the full-length (200 kDa) and cleaved form (100 kDa) of PTPµ were expressed (Fig. 4 a) as expected (12). PTPµ expression in MvLu cells is regulated by cell density (25). In our studies, the expression of PTPµ also increased with cell density (data not shown). However, at 50% and 90% confluence, PTPµ expression appeared to be approximately the same (Fig. 4 a). MvLu cell cultures were grown at these two densities to control the amount of cell contact. When MvLu cells are grown to 50% confluence there is little cell contact while at 90% confluence the majority of cells adhere to one another.

We used a retrovirus encoding antisense PTPµ (6) to infect MvLu cells. Immunoblot analysis demonstrated that the full-length protein (200 kDa band) was substantially reduced in cells infected with PTPµ antisense virus when compared to cells infected with control virus (Fig. 4 a, lane 5). The 100 kDa immunoreactive band was also reduced (Fig. 4 a, lane 5). These results confirmed that PTPµ antisense expression inhibited the new synthesis of PTPµ.

RACK1 was immunoprecipitated by antibodies to RACK1 (Fig. 4 c), but was not detected when a control mouse antibody was used for immunoprecipitation (Fig. 4 b). PTPµ was immunoprecipitated with a monoclonal antibody to the extracellular domain (BK2-Fig. 4 d) or with a polyclonal antibody to the C-terminus (471-Fig. 4e). When immunoprecipitates of PTPµ
were probed on immunoblots with anti-RACK1 antibody, an association was detected (Fig. 4 d-e). The association increased at high cell density (Fig. 4 d-e-lane 3). The interaction was not substantially altered when PKCs were stimulated by phorbol ester (PMA) treatment (Fig. 4 d-e-lane 4). However, when PTPμ expression was reduced in antisense-infected cells, it no longer interacted with RACK1 (Fig. 4 d-e-lane 5). These data suggest that endogenous RACK1 and PTPμ interact predominantly when cell-cell contact occurs.

**RACK1 localizes to points of cell-cell contact at high cell density**

PTPμ localizes to points of cell-cell contact in MvLu cells (12) and as shown in Fig. 5 (a-d). Immunocytochemical analysis of subconfluent cultures of MvLu cells is shown in Figure 5 (a-b). PTPμ localized to filopodial extensions that contacted between adjacent MvLu cells (Fig. 5 a and b). When cells were plated at higher density, PTPμ was restricted to points of cell-cell contact (Fig. 5 c and d). To determine the localization of RACK1, we performed immunocytochemistry on MvLu cells. The RACK1 protein is predominantly cytoplasmic in subconfluent MvLu cells (Fig. 5 e and f). As cell density increased RACK1 translocated to the plasma membrane. At high cell density, RACK1 also decorated points of cell-cell contact in MvLu cells (Fig. 5 g and h). We performed double-label immunocytochemistry on MvLu cells using antibodies to PTPμ (Fig. 6b) and RACK1 (Fig. 6c). The arrows in Figure 6 illustrate concentration of RACK1 and PTPμ at sites of cell-cell contact. The translocation of RACK1 to the membrane and points of cell-cell contact at high cell density is likely to be related to its increased association with PTPμ (Fig. 4).
We then used a retrovirus encoding antisense PTPµ to infect MvLu cells and performed immunocytochemistry. There was a dramatic morphological change in the MvLu cells when infected with the antisense PTPµ retrovirus (Fig. 7). The antisense infected cells were never able to grow to high cell density. However, cell-cell contact sites were still present as evidenced by localization of cadherins (Fig. 7 a and b). When PTPµ expression was reduced, RACK1 no longer localized to the plasma membrane or points of cell-cell contact (Fig. 7 c and d). These data suggest that the PTPµ protein plays a role in recruiting RACK1 to points of cell-cell contact in MvLu cells.

**Discussion**

In this study, we used the yeast two-hybrid screen to isolate PTPµ interacting proteins. We identified an interaction between the membrane-proximal catalytic domain of PTPµ (PTPµD1) and RACK1 (receptors for activated protein C kinase). Since yeast do not have traditional tyrosine kinases, the interaction of PTPµD1 and RACK1 was likely to be mediated by protein-protein interactions and not dependent upon phosphotyrosine. We characterized the association between RACK1 and PTPµD1 using the recombinant baculovirus expression system, and have shown that the intracellular segment of PTPµ binds to RACK1 in insect cells. The RACK1/PTPµ interaction was disrupted by a constitutively active src PTK. These data suggest that RACK1/src and RACK1/PTPµ may form mutually exclusive signaling complexes. In addition, we showed an association between PTPµ and RACK1 using MvLu cells, which express both proteins endogenously. The interaction of PTPµ and RACK1 was not affected by phorbol ester stimulation of PKC, suggesting that when RACK1 is bound to PTPµ it is still likely to be
able to bind PKC. PTPµ expression increases with increasing cell density in MvLu cells (25). At high cell density, we observed an increased association of RACK1 with PTPµ as well as increased translocation of RACK1 to the plasma membrane and points of cell-cell contact. In cells expressing antisense PTPµ, RACK1 remained cytoplasmic suggesting that PTPµ may play a role in recruiting RACK1 to cell contact sites. Together these data suggest that PTPµ and RACK1 form a signaling complex at high cell density.

RACK1 is a homolog of the β subunit of heterotrimeric G proteins and is composed of WD repeats (16). Both Gβ and RACK1 form seven propeller structures (seven independently folding loops) proposed to mediate protein-protein interactions (17). RACK1 was originally identified as a protein that binds to activated protein kinase C (PKC) (16). It has been suggested that activated PKC binding to RACK1 is required for the translocation of the enzyme to the plasma membrane, its physiologically relevant site of action (15). In addition, RACK1 seems to serve as a general scaffolding protein for a number of signaling enzymes including src (20).

Receptor protein tyrosine phosphatases are involved in cell adhesion (1). PTPµ has been shown to induce cell adhesion by homophilic binding (2,3,5). In addition, it also appears to regulate cadherin-mediated cell adhesion by binding to the cadherin/catenin complex (6,12,13). The PTPµ/RACK1 interaction appears to occur predominantly in cells at high cell density. These data indicate that the PTPµ/RACK1 interaction may be induced by cell-cell contact. Based on our antisense experiments, it is clear that the interaction requires the presence of the PTPµ protein. Our hypothesis is that the PTPµ/RACK1 interaction is likely to be induced by cell adhesion, which may recruit other signaling proteins that are important for PTPµ-dependent
One could speculate that PKC or other signaling molecules associated with RACK1 might be downstream of PTPµ-dependent signals induced by cell-cell adhesion.

If the association of RACK1 and PTPµ brings activated PKC close to its site of action at the membrane, how might other cell-cell adhesion molecules, like cadherins, be involved? There have been suggestions in the literature that PKC may regulate E-cadherin-dependent adhesion. Adherens junctions serve to anchor the actin cytoskeleton at regions of cell-cell contact. Investigators have postulated that the cytoskeletal reorganization which occurs during the formation of adherens junctions is induced by PKC activation and that PKC, in turn, may regulate cadherin-dependent adhesion (30). Clearly our data suggest an interesting relationship exists between PKC signal transduction mechanisms and the PTPµ cell-cell adhesion molecule. It is likely that the interaction of PTPµ and RACK1 at high cell density recruits PKC or other RACK1 binding partners to sites of cell-cell contact to transduce adhesion-dependent signals.

Tyrosine phosphorylation by the src PTK negatively regulates cadherin-dependent adhesion (7,31,32); although the mechanism is unknown. Previously, we tested the ability of the src tyrosine kinase to regulate PTPµ/cadherin interactions. We used a series of WC5 cell lines, which express PTPµ endogenously, and ectopically expressed E-cadherin. We analyzed the effect of tyrosine phosphorylation on the composition of the PTPµ/cadherin complex and our data suggested that increased tyrosine phosphorylation of E-cadherin resulted in decreased association with PTPµ (13). Interestingly, RACK1 binds to the src PTK. In this study, we found that constitutively active src disrupts the binding between PTPµ and RACK1. Since src is known to negatively regulate cadherin-dependent adhesion, the ability of PTPµ and src to bind
RACK1 may directly affect tyrosine phosphorylation of the cadherin complex via PTPµ or indirectly by regulating the presence of the src PTK in the complex. Together, these data suggest that PTPµ may be altering src signaling pathways via its interaction with RACK1.

Since RACK1 binds to the conserved PTP catalytic domain of PTPµ, a number of other PTPs may also interact with RACK1. It is interesting to note that many PTPs are known to regulate the src cytoplasmic PTK (1). Importantly, RACK1 is known to bind to c-src and inhibit its tyrosine kinase activity (20). Based upon the results from that study, c-src does not appear to phosphorylate native RACK1 (20). Our data suggest that one of the links between PTPs and src PTK signaling may be the RACK1 protein. This manuscript demonstrates that PTPµ association with RACK1 is altered in the presence of src, suggesting that there may be mutually exclusive interactions of src and PTPµ with RACK1. One caveat of these studies is that they were done using a constitutively active src PTK. However, we believe that the PTP versus PTK competition for binding to RACK1 may be a common form of regulation for signaling complexes. For example, protein-protein interactions with scaffolding molecules such as RACK1 may control tyrosine phosphorylation of substrate proteins via their mutually exclusive interactions with a tyrosine kinase or phosphatase. More importantly, the ability of src and PTPµ to compete for RACK1 binding may be an important mechanism for regulation of cell-cell adhesion and signal transduction.
Acknowledgments

A number of individuals provided assistance with this study, and their efforts were greatly appreciated including Dr. Leif Stordal, Rachna Dave, Carol Luckey, Dr. Sandra Lemmon’s lab and Dr. Hsing Jien Kung’s lab especially L. Ravi. We would also like to thank Dr. Vance Lemmon, Dr. Hsing Jien Kung, Dr. Steven Reeves, Dr. Jonathan Cooper, Dr. Michael Weber and Dr. David Morgan for reagents. We would also like to thank Dr. Carole Leidtke for reagents and helpful suggestions. This work was supported by a grant from the American Cancer Society, Ohio Division, Cuyahoga County Unit to S.B.K.; NIH grant (1RO1-EY12251) to S.B.K. This research, under DAMD17-98-1-8586, was supported by the Department of Defense Prostate Cancer Research Program, which is managed by the U.S. Army Medical Research and Materiel Command. C. Hellberg was supported by The Swedish Society for Medical Research. Additional support was provided by the Visual Sciences Research Center Core Grant from the National Eye Institute (PO-EY11373).
References


Figure Legends

Figure 1. PTPµ and RACK1 interact in yeast. Panel a illustrates the growth of two yeast clones containing both RACK1 (R) and PTPµ (µ) grown on medium containing galactose or glucose. Panel b shows the same PTPµ/RACK1 (R&µ) clones grown on medium containing Xgal. Positive (+) and negative (-) controls are described in the materials and methods section. The yeast strain containing PTPµ, RACK1 and src (R&µ&src) is also shown.

Figure 2. PTPµ and RACK1 interact in Sf9 cells. Panels a-c are immunoprecipitations that were separated by 7.5% or 10% SDS-PAGE and probed with monoclonal antibodies to either the HA tag of RACK1 (a and b), intracellular domain of PTPµ (c). Panel a demonstrates that equal amounts of RACK1 are present in RACK1 immunoprecipitates from Sf9 cells infected with either RACK1 (lane 1), RACK1 and PTPµ (lane 3), RACK1/PTPµ plus PMA (lane 4). Cells infected with PTPµ only (panel a, lane 2), display no detectable RACK1. Panel b illustrates immunoblots of PTPµ immunoprecipitates probed with HA antibody to detect the RACK1 protein. PTPµ and RACK1 interact only when they are co-expressed regardless of the presence or absence of PMA (panel b, lanes 3-4). The bar to the left of panels a and b represents the 52kDa molecular weight marker. Recombinant RACK1 migrates at this molecular weight due to the addition of various tags. Panel c shows an immunoblot of PTPµ immunoprecipitates that were separated by 7.5% SDS-PAGE and probed with a monoclonal antibody to PTPµ (SK15). In all Sf9 cells infected with PTPµ, equal amounts of PTPµ are present. Cells infected with RACK1 only (lane 1), display no detectable PTPµ. The bar in panel c represents the 91kDa molecular weight marker.
Figure 3. The interaction between RACK1 and PTP\(\mu\) is disrupted by constitutively active src. Immunoprecipitates are shown from Sf9 cells infected with either RACK1 (lane 1), RACK1 and PTP\(\mu\) (lane 2), RACK1/PTP\(\mu\)/src (lane 3) and RACK1/PTP\(\mu\)/src in the presence of the src tyrosine kinase inhibitor PP2 (lane 4). Panel a demonstrates that equal amounts of RACK1 are present in RACK1 immunoprecipitates. Panel b illustrates immunoblots of PTP\(\mu\) immunoprecipitates probed with HA antibody to detect the RACK1 protein. Panel c shows an immunoblot of PTP\(\mu\) immunoprecipitates that were separated by 7.5% SDS-PAGE and probed with a monoclonal antibody to PTP\(\mu\) (SK15). An immunoblot of lysates using a src antibody demonstrates that src is expressed only in cells infected with the src virus (panel d). An immunoblot of lysates using an anti-phosphotyrosine antibody demonstrates that src is inhibited in cells infected with the src virus in the presence of the PP2 src inhibitor (panel e).

Figure 4. Endogenous RACK1 interacts with endogenous PTP\(\mu\) and the interaction is not altered by PMA treatment. MvLu cells were grown to 50% (lanes 1-2) or 90% confluence (lanes 3-5) or MvLu cells were treated with phorbol esters (PMA). Lysates from MvLu cells were immunoprecipitated with control (b), RACK1 (c) or PTP\(\mu\) (d, e) antibodies as indicated and were separated by 10% or 6% SDS-PAGE. Panel a is an immunoblot of lysates using an antibody to PTP\(\mu\) (SK15). Panels b-e are immunoblots of the immunoprecipitates probed with antibody to RACK1. The control antibody (8D9) did not immunoprecipitate RACK1 (panel b). RACK1 was readily detectable in all RACK1 immunoprecipitates (panel c). PTP\(\mu\) interacted with RACK1 predominantly in 90% confluent MvLu cells (panel d and e). Importantly, the interaction was not substantially altered by PMA treatment (panel d and e). Finally, the interaction between PTP\(\mu\)
and RACK1 was abolished by down-regulating PTPµ expression using a virus encoding antisense PTPµ (panel d and e-lane 5). The molecular weight markers in panel a represent the 208 and 130kDa markers, respectively. The arrows in each panel indicate the 36kDa RACK1 band.

Figure 5. Immunocytochemical localization of PTPµ and RACK1 in MvLu cells. Phase contrast (panels a, c, e and g) or fluorescence (panels b, d, f and h) images of MvLu cells are shown. Cells labeled with antibodies to PTPµ (SK15-panels a-d) show that PTPµ is localized to filopodial extensions of the cells and points of cell-cell contact in subconfluent MvLu cells (panels a and b). When the cells were plated at higher density, PTPµ was localized to points of cell-cell contact (panels c and d). RACK1 (panels e-h) was localized in the cytoplasm at low cell density (panels e and f). At high cell density, RACK1 was localized to points of cell-cell contact (panels g and h). Scale bar = 30µm.

Figure 6. Double-label immunocytochemistry of RACK1 and PTPµ in confluent MvLu cells. Phase contrast (panel a) or fluorescence (panels b and c) images of MvLu cells are shown. Cells labeled with antibodies to PTPµ (panel b) show that PTPµ is localized points of cell-cell contact. RACK1 (panel c) was also localized to points of cell-cell contact. Arrows indicate some of the areas of co-localization. Scale bar = 20µm.

Figure 7. Immunocytochemical localization of cadherins and RACK1 in MvLu cells infected with an antisense PTPµ-encoding retrovirus. A retrovirus encoding antisense PTPµ was used to infect MvLu cells and immunocytochemistry was performed. When PTPµ expression was
reduced, some cell-cell contacts sites were still present as evidenced by localization of classical cadherins using the pan cadherin antibody (Fig. 7 a and b). However, RACK1 no longer localized to the plasma membrane and points of cell-cell contact (Fig. 7 c and d). These data suggest that the PTPµ protein plays a role in recruiting RACK1 to points of cell-cell contact in MvLu cells. Scale bar = 20µm.
RACK immunoblots

a. RACK IPs

RACK + ++ +
PTPμ-intra + ++ +
PMA +

b. PTPμ IPs

+ + ++ +

PTPμ immunoblots

c. PTPμ IPs

+ + ++ +

RACK immunoblots

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<th>b. PTPµ IPs</th>
<th>c. PTPµ IPs/PTPµ blot</th>
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Lysates
a. PTPµ immunoblot

b. Control
c. RACK1
d. PTPµ (extra Ab)
e. PTPµ (intra Ab)
The PTPmu protein tyrosine phosphatase binds and recruits the scaffolding protein RACK1 to cell-cell contacts
Tracy Mourton, Carina B. Hellberg, Susan M. Burden-Gulley, Jason Hinman, Amy Rhee and Susann M. Brady-Kalnay

*J. Biol. Chem.* published online February 13, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010823200

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