Lack of Association between Receptor Protein Tyrosine Phosphatase RPTPμ and Cadherins
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Abstract. RPTPμ is a receptor-like protein tyrosine phosphatase that mediates homophilic cell-cell interactions. Surface expression of RPTPμ is restricted to cell-cell contacts and is upregulated with increasing cell density, suggesting a role for RPTPμ in contact-mediated signaling. It was recently reported (Brady-Kalnay, S.M., D.L. Rimm, and N.K. Tonks. 1995. J. Cell Biol. 130:977-986) that RPTPμ binds directly to cadherin/catenin complexes, and thus may regulate the tyrosine phosphorylation of such complexes. Here we report that this concept needs revision. Through reciprocal precipitations using a variety of antibodies against RPTPμ, cadherins, and catenins, we show that RPTPμ does not interact with cadherin/catenin complexes, even when assayed under very mild lysis conditions. We find that the anti-RPTPμ antiserum used by others precipitates cadherins in a nonspecific manner independent of RPTPμ. We conclude that, contrary to previous claims, RPTPμ does not interact with cadherin complexes and thus is unlikely to directly regulate cadherin/catenin function.

Receptor-like protein tyrosine phosphatases (receptor PTPases) constitute a relatively new family of transmembrane proteins that are thought to transduce extracellular signals by dephosphorylating phosphotyrosine residues on cytosolic substrates. By counterbalancing the actions of protein tyrosine kinases, the receptor PTPs are thought to have important roles in regulating cell proliferation and/or differentiation (for review see Charbonneau and Tonks, 1992; Walton and Dixon, 1993). The receptor PTPase receptor-like protein tyrosine phosphatase (RPTPμ) contains two intracellular phosphatase domains and a modular ectodomain consisting of four fibronectin type III-like repeats, a single immunoglobulin-like domain, and an NH₂-terminal MAM domain (Gebbink et al., 1991; Beckmann and Bork, 1993). We and others previously showed that the ectodomain of RPTPμ can mediate homophilic cell-cell interactions (Gebbink et al., 1993; Brady-Kalnay et al., 1993). Homophilic binding by RPTPμ is independent of its catalytic activity but requires both the immunoglobulin-like domain (Brady-Kalnay et al., 1994) and the MAM domain (Zondag et al., 1995). Immunofluorescent analysis in subconfluent cells shows that RPTPμ is concentrated in regions of close cell-cell contact. Furthermore, we showed that cell surface expression of RPTPμ is upregulated with increasing cell density (Gebbink et al., 1995). Taken together, the available evidence strongly suggests that RPTPμ is involved in contact-mediated signaling.

To date, the physiological substrates of RPTPμ are not known. Identification of these substrates is hampered by the fact that most PTPases show promiscuous activity towards tyrosine-phosphorylated proteins, and that their high basal enzymatic activity does not seem to be tightly regulated. Instead, it has been proposed that the cellular action of receptor PTPases such as RPTPμ is determined by their location on the cell surface: contact-induced clustering of RPTPμ is thought to bring the catalytic domain into proximity with specific substrates, which will then trigger intracellular signaling (Gebbink et al., 1995). Hence, membrane-associated proteins in regions of close cell-cell contacts, such as adherens and/or tight junctions, are candidate substrates.

Using communoprecipitation and immunofluorescence assays, Brady-Kalnay et al. (1995) recently reported in vivo association of RPTPμ with cadherins and catenins in mink lung cells and in rat tissue lysates. Based on their findings, the authors suggested that RPTPμ binds directly to more than 80% of cadherin and thereby may regulate the tyrosine phosphorylation, and thus function, of the cadherin/catenin complex in vivo (Brady-Kalnay et al., 1995). The potential importance of this signaling principle prompted us to expand on these findings. Using a panel of different monoclonal antibodies against RPTPμ as well as antibodies to cadherins and catenins, and using diverse lysis protocols, we have reexamined the putative RPTPμ-cadherin association in various cell systems. The results ob-

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I. Abbreviations used in this paper: GST, glutathione-S-transferase; PTPase, protein tyrosine phosphatase; RPTP, receptor-like protein tyrosine phosphatase.
tained contradict the earlier findings by Brady-Kalnay et al. (1995). Our data indicate that RPTPµ does not interact with cadherins or catenins and thus is unlikely to regulate their function in a direct manner.

Materials and Methods

Cells and Antibodies

Mv1Lu mink lung epithelial and COS cells were cultured in DMEM supplemented with 8% FCS and antibiotics. The generation and characterization of monoclonal antibodies 1E1 (isotype IgG1/k) and 3D7 (IgG2a/s) directed against the RPTPµ ectodomain has been described (Gebbink et al., 1993). Monoclonal antibodies 1D5 (IgG1/s) and 2B11 (IgG2a/s) are derived from the same hybridoma fusion as the monoclonals described above and generated accordingly. Ascoric fluid containing monoclonal antibody BK2, raised against a synthetic peptide (Brady-Kalnay et al., 1993), was kindly provided by Dr. N. Tonks (Cold Spring Harbor, NY). Polyclonal antiserum Ab37, raised against a peptide corresponding to the COOH terminus of RPTPµ and monoclonal antibody 3G4, directed against the first fibronectin domain, have been described (Gebbink et al., 1991). Monoclonal Pan-cadherin antibody was purchased from Sigma Chem. Co. (St. Louis, MO). Polyclonal anti-cadherin antibodies, raised against a fusion protein between glutathione-S-transferase and the intracellular domain of E-cadherin were kindly provided by Drs. P. Bringuier and J. Schalken (University Hospital Nijmegen, The Netherlands). Monoclonal antibody to p120<sup>ca</sup> was purchased from Transduction Laboratories (Lexington, KY). Polyclonal antibodies against α- and β-catenin were kindly provided by Dr. R. Kypri (U.C. San Francisco, CA) and Drs. O. Huber and R. Kemler (Max Planck Institute, Freiburg, Germany).

COS Cell Transfections

COS cells were transfected in 60-cm<sup>2</sup> culture dishes using a modified DEAE-dextran method. In brief, 60% confluent COS cells were washed and overlaid with a mixture containing 500 µg/ml DEAE-dextran and 5 µg GMt2-fHµ plasmid DNA (Gebbink et al., 1993) in PBS for 30 min. Cells were then incubated in DMEM supplemented with 8% FCS and 80 µM chloroquine for 3 h. Finally, cells were shocked in 10% DMSO and cultured in fresh medium. 2 d after transfection, COS cells were lysed and immunoprecipitations were performed according to Brady-Kalnay et al. (1995).

Immunoprecipitation and Immunoblotting

Cells were washed once in PBS and scraped on ice in lysis buffer containing 20 mM Tris, pH 7.6, 1% Triton X-100, 5 mM EDTA supplemented with 5 µg/ml leupeptin, 5 µg/ml aprotinin, 10 µM Pefabloc (Fluka Chemie AG, Switzerland), 200 µM phenylarsenic oxide, 1 mM sodium orthovanadate, and 0.1 mM sodium molybdate. Triton-insoluble material was pelleted by centrifugation at 5,000 g for 5 min. Supernatant was incubated with specific antibodies precoupled to Protein A-Sepharose beads (Phar-}

Results and Discussion

Analysis of RPTPµ Immunoprecipitates

To examine whether RPTPµ and cadherins may physically interact, we used various antibodies in immunoprecipi-

Figure 1. Examination of the putative RPTPµ-cadherin interaction in Mv1Lu mink lung cells. (A) Analysis of RPTPµ-immuno-

precipitates for the presence of cadherins. Monoclonal antibodies recognizing different epitopes were used to immunoprecipitate RPTPµ from Mv1Lu cells. Immunoprecipitates were analyzed on immunoblots using either anti-RPTPµ monoclonal antibody 3G4 (upper panel) or polyclonal anti-cadherin antibody (lower panel). Lane 7 contains total cell lysate (T.L.), lanes 2-6 immunoprecipitates using different anti-RPTPµ monoclonals, and lane 7 contains an immunoprecipitation using monoclonal anti-Pan cadherin antibody. In the upper panel, RPTPµ is visible in total lysate only after prolonged exposure. The <i>~</i>180-kD protein band present in the 1E1 and BK2 precipitations represents some nonspecific background signal. Note that the anti-Pan cadherin antibody does not coprecipitate any RPTPµ, and that of the five anti-RPTPµ monoclonals tested, only BK2 coprecipitates cadherins. (B) Analysis of cadherin/catenin complexes for the presence of RPTPµ. Cadherin complexes were immunoprecipitated using antibodies to cadherins, p120<sup>ca</sup>, or α- and β-catenin, as indicated. As a control, RPTPµ was immunoprecipitated using antibody 3D7. The immunoprecipitates were analyzed on immunoblots using antibody 3G4 against RPTPµ, and various antibodies to cadherins, p120, and α- and β-catenin, respectively. It is seen that no RPTPµ is detectable in cadherin complexes precipitated by either anti-cadherin or anti-catenin antibodies. Conversely, no cadherins or catenins are present in the RPTPµ immunoprecipitates.
or 90–100-kD region, where cadherins and catenins should migrate (Gebbink et al., 1995; Zondag, G.C., and M.F. Gebbink, unpublished results). Since various lysis conditions were tested, including very mild digitonin and low-salt buffers, it is unlikely that the lack of interactions is due to inappropriate conditions. An alternative explanation is that antibody 3D7 may somehow interfere with the interaction between RPTPμ and associating proteins. Therefore, we tested four other monoclonal antibodies recognizing different epitopes in the RPTPμ ectodomain, including the BK2 antibody used by Brady-Kalnay et al. (1995). In addition, we used an anti-Pan cadherin monoclonal antibody to test for coprecipitation of RPTPμ (Fig. 1 A).

Lysis of Mv1Lu cells and immunoprecipitations were done exactly as reported by Brady-Kalnay et al. (1995). Fig. 1 A (upper panel) shows a Western blot of the RPTPμ immunoprecipitates probed with anti-RPTPμ monoclonal antibody 3G4. As can be seen from lanes 2 to 6, all anti-RPTPμ monoclonals precipitate a 200-kD protein corresponding to full-length RPTPμ and the 100-kD cleaved form of RPTPμ (Gebbink et al., 1995). In contrast, no RPTPμ is detected in the cadherin-immunoprecipitate (Fig. 1 A, upper panel, lane 7). In the lower panel of Fig. 1 A, the immunoblot was reprobed with polyclonal anti-Pan cadherin antibody; this antiserum recognizes two bands in total cell lysates (lane I; T.L.). The anti-cadherin antibody only precipitates the upper band (130 kD) of this doublet (lower panel, lane 7), as was also observed by Brady-Kalnay et al. (1995). When analyzing the RPTPμ immunoprecipitations by various monoclonal antibodies (lanes 2–6), it is seen that only the BK2 antibody is able to precipitate catenins. None of the other anti-RPTPμ monoclonals coprecipitates any cadherins, although they all recognize a different epitope on RPTPμ. This makes it very unlikely that the antibodies used would interfere with a putative RPTPμ-cadherin interaction.

### Analysis of Cadherin/Catenin Complexes

Intracellularly, cadherins associate with catenins to provide a link to the actin cytoskeleton (for review see Cowin, 1994). In addition, cadherin complexes contain the β-catenin–related protein p120cas, a phosphotyrosine substrate of tyrosine kinases (Reynolds et al., 1994; Shibamoto et al., 1995). To further examine the putative interaction between RPTPμ and cadherin complexes, we probed cadherin/catenin immunoprecipitates with anti-RPTPμ antibody and vice versa. As shown in Fig. 1 B, no RPTPμ is detectable in cadherin complexes immunoprecipitated with antibodies against cadherin, α- and β-catenin, or p120cas. Control experiments show that these antibodies do precipitate their respective antigens, and that antibodies to α-catenin, β-catenin, and p120cas do coprecipitate cadherins. Conversely, no cadherin or catenin/p120cas proteins are detectable in anti-RPTPμ immunoprecipitates (Fig. 1 B; see also Fig. 1 A). These results reinforce the notion that there is no physiological interaction between RPTPμ and cadherin complexes.

### Overexpression of RPTPμ Does Not Induce Cadherin Association

We next tried to induce RPTPμ-cadherin association by overexpressing RPTPμ in COS cells. COS-7 cells, which lack endogenous RPTPμ, were transfected with either empty vector or RPTPμ cDNA. Transfected COS cells were analyzed by immunoprecipitation using anti-RPTPμ antibodies 3D7 and BK2, and anti-Pan cadherin antibody. As expected, both 3D7 and BK2 precipitate RPTPμ only from RPTPμ-expressing cells (Fig. 2, left panel). However, despite the high RPTPμ expression levels, anti-cadherin antibody fails to coprecipitate any RPTPμ. Overexposure of the same immunoprecipitates probed with polyclonal anti-cadherin antibody (Fig. 2, right panel) shows that the

| IP: 3D7 | BK2 | α-Cadherin | 3D7 | BK2 | α-Cadherin | T.L. | 3D7 | BK2 | α-Cadherin | T.L. |
|--------|-----|------------|-----|-----|------------|-----|-----|-----|------------|-----|
| 1      | 2   | 3          | 4   | 5   | 6          | 7   | 8   | 1   | 2          | 3   |
| blot: anti-RPTPμ | blot: anti-Pan cadherin |

*Figure 2. Overexpression of RPTPμ does not result in association with endogenous cadherins. COS cells were transfected with either empty vector (lanes 1, 2, 3, and 7, as indicated by a minus) or with RPTPμ cDNA (lanes 4, 5, 6, and 8, as indicated by a plus) and lysed after 2 d. Cell lysates were analyzed for RPTPμ/cadherin complexes by immunoprecipitation using the anti-RPTPμ antibodies BK2 and 3D7, and anti-Pan cadherin antibody. As expected, both 3D7 and BK2 precipitate RPTPμ only from RPTPμ-expressing cells (Fig. 2, left panel). However, despite the high RPTPμ expression levels, anti-cadherin antibody fails to coprecipitate any RPTPμ. Overexposure of the same immunoprecipitates probed with polyclonal anti-cadherin antibody (Fig. 2, right panel) shows that the*
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subsequent precipitation with BK2 antibody does not

viturally no RPTPtx can be precipitated anymore. As expected, subsequent precipitation with BK2 antibody does not bring down any RPTPtx.

Fig. 3 B shows the same series of immunoprecipitations probed with anti-cadherin antibody. As suspected from the COS cell experiments, BK2 can still precipitate cadherins from mink cell lysates, consistent with BK2 acting nonspecifically. As an additional control, we used polyclonal antiserum 37 raised against the RPTPtx COOH terminus, which recognizes all forms of RPTPtx (i.e., cleaved, uncleaved, glycosylated, and nonglycosylated forms; Gebbink et al., 1995). As shown in Fig. 3 C, antibody 37 does not precipitate any RPTPtx from the 3D7-precleared lysates, demonstrating that RPTPtx depletion was complete. This indicates that the BK2 antibody directly recognizes an epitope on cadherins and that it precipitates cadherins independently of RPTPtx.

Nonspecific Precipitation of Cadherins by BK2

To further examine the observed reactivity of BK2 against cadherins, we depleted RPTPtx from MvlLu cell lysates by three subsequent precipitations with antibody 3D7 (Fig. 3), and then precipitated the depleted lysates using antibody BK2. Fig. 3 A shows an immunoblot of the immunoprecipitates probed with anti-RPTPtx antibody. It is seen that a single 3D7 precipitation brings down ~90% of all RPTPtx from the lysate; after two subsequent precipitations, virtually no RPTPtx can be precipitated anymore. As expected, subsequent precipitation with BK2 antibody does not bring down any RPTPtx.

Concluding Remarks

In the present study, we have examined the putative association between RPTPtx and cadherins. We found no evidence for such an interaction. In particular, our experiments reveal that the reported RPTPtx/cadherin association (Brady-Kalnay et al., 1995) is due to the use of a nonspecific antibody which cross-reacts with cadherins. Brady-Kalnay et al. (1995) also performed overlay experiments, where a glutathione-S-transferase (GST)-E cadherin fusion protein was transferred onto nitrocellulose and shown to bind to soluble GST-RPTPtx. Using recombinant baculovirus, we have generated native fusion proteins consisting of GST fused to the complete intracellular domain of RPTPtx. In similar overlay assays using total mink cell lysates instead of purified proteins, we were unable to detect binding of the fusion protein to cadherins present in the transfected total cell lysate (data not shown). The discrepancy with the reported overlay results may reside in the fact that Brady-Kalnay et al. (1995) produced their cadherin and RPTPtx fusion proteins in bacteria, which is likely to yield misfolded or denatured protein. Moreover, the bacterial products were applied in rather large amounts, with a high risk of nonspecific protein–protein interactions.

That RPTPtx does not associate with cadherins or with catenins is supported by preliminary analysis of RPTPtx knockout mice (Gebbink, M.F., E. Feiken, G.C. Zondag, and W.H. Moolenaar, manuscript in preparation). In these RPTPtx-deficient mice, β-catenin tyrosine phosphorylation patterns are unaltered when compared to wild-type mice and furthermore, no cadherin-associated phenotype is observed. Identification of the physiological substrate(s) of RPTPtx thus remains a challenge for future studies.

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3D7 immunoprecipitate lacks detectable cadherin. Surprisingly, however, the BK2 antibody precipitates small amounts of cadherin from both RPTPtx-deficient control cells and RPTPtx-overexpressing cells. It thus appears that the BK2 antibody acts in a nonspecific manner, as it precipitates cadherins independently of RPTPtx. These results show that, even after high overexpression, RPTPtx fails to associate with cadherins.

Figure 3. Monoclonal antibody BK2 precipitates cadherins independently of RPTPtx. MvlLu cell lysate was depleted from RPTPtx by three consecutive immunoprecipitations using antibody 3D7 (lanes 1, 2 and 3, respectively). Half of the depleted lysate was subsequently analyzed by immunoprecipitation using the BK2 antibody. In A, total lysate and immunoprecipitations are probed with anti-RPTPtx antibody 3G4. Note that no RPTPtx is detectable in the BK2 immunoprecipitate after three consecutive immunoprecipitations with 3D7. B shows identical samples probed with polyclonal anti-Pan cadherin antibody. As can be seen, BK2 still immunoprecipitates cadherins from mink cell lysates after depletion of RPTPtx. (C) No residual RPTPtx is present after three consecutive 3D7-immunoprecipitations. Half of the RPTPtx-depleted lysate was used above was supplemented with an equal volume of 2× BUSS buffer. Polyclonal antibody 37, raised against a COOH-terminal RPTPtx peptide, was then used to precipitate any residual RPTPtx which may have been unrecognized by the 3D7 antibody. No RPTPtx is detectable in the Ab 37 precipitate, confirming complete RPTPtx depletion of the mink cell lysates.
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