

Lack of Association between Receptor Protein Tyrosine Phosphatase RPTP μ and Cadherins

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Abstract. RPTP μ is a receptor-like protein tyrosine phosphatase that mediates homophilic cell-cell interactions. Surface expression of RPTP μ is restricted to cell-cell contacts and is upregulated with increasing cell density, suggesting a role for RPTP μ 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 RPTP μ 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 RPTP μ , cadherins, and catenins, we show that RPTP μ does not interact with cadherin/catenin complexes, even when assayed under very mild lysis conditions. We find that the anti-RPTP μ antiserum used by others precipitates cadherins in a nonspecific manner independent of RPTP μ . We conclude that, contrary to previous claims, RPTP μ does not interact with cadherin complexes and thus is unlikely to directly regulate cadherin/catenin function.

RECEPTOR-LIKE protein tyrosine phosphatases (receptor PTPases)¹ 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 (RPTP μ) 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 RPTP μ can mediate homophilic cell-cell interactions (Gebbink et al., 1993; Brady-Kalnay et al., 1993). Homophilic binding by RPTP μ 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 RPTP μ is concentrated in regions of close cell-cell contact. Furthermore, we showed that cell surface expression

of RPTP μ is upregulated with increasing cell density (Gebbink et al., 1995). Taken together, the available evidence strongly suggests that RPTP μ is involved in contact-mediated signaling.

To date, the physiological substrates of RPTP μ 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 RPTP μ is determined by their location on the cell surface: contact-induced clustering of RPTP μ 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 coimmunoprecipitation and immunofluorescence assays, Brady-Kalnay et al. (1995) recently reported in vivo association of RPTP μ with cadherins and catenins in mink lung cells and in rat tissue lysates. Based on their findings, the authors suggested that RPTP μ 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 to RPTP μ as well as antibodies to cadherins and catenins, and using diverse lysis protocols, we have reexamined the putative RPTP μ -cadherin association in various cell systems. The results ob-

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1. *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 RPTP μ 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/ λ) and 3D7 (IgG2a/ κ) directed against the RPTP μ ectodomain has been described (Gebbink et al., 1995). Monoclonal antibodies 1D5 (IgG1/ κ) and 2B11 (IgI2a/ κ) are derived from the same hybridoma fusion as the monoclonals described above and generated accordingly. Ascites 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 RPTP μ 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^{cas} was purchased from Transduction Laboratories (Lexington, KY). Polyclonal antibodies against α - and β -catenin were kindly provided by Dr. R. Kypta (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-cm² culture dishes using a modified DEAE-dextran method. In brief, 60% confluent COS cells were washed with PBS and overlaid with a mixture containing 500 μ g/ml DEAE-dextran and 5 μ g pMT2-hFL μ plasmid DNA (Gebbink et al., 1993) in PBS for 30 min. Cells were then incubated in DMEM supplemented with 8% FCS and 80 μ M 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 μ g/ml leupeptin, 5 μ g/ml aprotinin, 10 μ M Pefabloc (Fluca Chemie AG, Switzerland), 200 μ M phenylarsene 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 (Pharmacia) for 4 h. Immune complexes were washed four times in lysis buffer and boiled in SDS sample buffer for 5 min. When using polyclonal antibody Ab37, an equal volume of 2 \times BUSS buffer (40 mM Tris, pH 7.6, 300 mM NaCl, 2% NP-40, 1% SDS, 2% deoxycholate, and 2 mM DTT) was added to the Triton-soluble fraction before immunoprecipitation. Ab37 immunocomplexes were washed four times in 1 \times BUSS buffer. Total lysate was prepared by adding 4 \times SDS sample buffer to the Triton-soluble cell lysate. Protein samples were analyzed on 8% SDS-PAGE gels followed by immunoblotting. Immunoblots were blocked in 5% nonfat dry milk in TBST (50 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) and incubated with specific antibodies as indicated. Appropriate secondary antibodies conjugated to horseradish peroxidase were detected by chemiluminescence (ECL, Amersham Intl., Buckinghamshire, UK).

Results and Discussion

Analysis of RPTP μ Immunoprecipitates

To examine whether RPTP μ and cadherins may physically interact, we used various antibodies in coimmunoprecipitation assays.

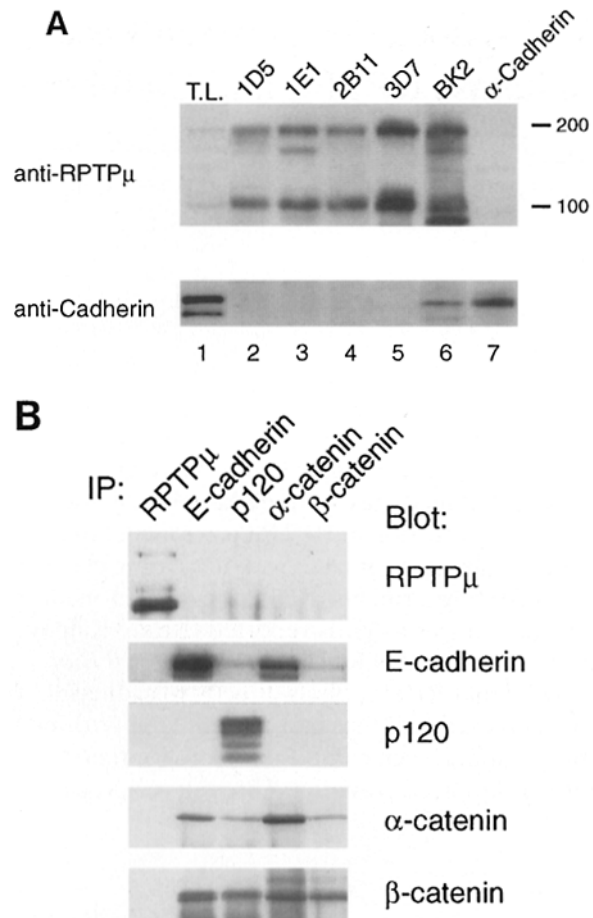


Figure 1. Examination of the putative RPTP μ -cadherin interaction in Mv1Lu mink lung cells. (A) Analysis of RPTP μ -immunoprecipitates for the presence of cadherins. Monoclonal antibodies recognizing different epitopes were used to immunoprecipitate RPTP μ from Mv1Lu cells. Immunoprecipitates were analyzed on immunoblots using either anti-RPTP μ monoclonal antibody 3G4 (upper panel) or polyclonal anti-cadherin antibody (lower panel). Lane 1 contains total cell lysate (T.L.), lanes 2–6 immunoprecipitates using different anti-RPTP μ monoclonals, and lane 7 contains an immunoprecipitation using monoclonal anti-Pan cadherin antibody. In the upper panel, RPTP μ is visible in total lysate only after prolonged exposure. The \sim 180-kD protein band present in the 1E1 and BK2 precipitations represents some non-specific background signal. Note that the anti-Pan cadherin antibody does not coprecipitate any RPTP μ , and that of the five anti-RPTP μ monoclonals tested, only BK2 coprecipitates cadherins. (B) Analysis of cadherin/catenin complexes for the presence of RPTP μ . Cadherin complexes were immunoprecipitated using antibodies to cadherins, p120^{cas}, or α - and β -catenin, as indicated. As a control, RPTP μ was immunoprecipitated using antibody 3D7. The immunoprecipitates were analyzed on immunoblots using antibody 3G4 against RPTP μ , and various antibodies to cadherins, p120, and α - and β -catenin, respectively. It is seen that no RPTP μ is detectable in cadherin complexes precipitated by either anti-cadherin or anti-catenin antibodies. Conversely, no cadherins or catenins are present in the RPTP μ immunoprecipitates.

In previous experiments, we used monoclonal antibody 3D7 to precipitate RPTP μ from surface-iodinated or metabolically labeled Mv1Lu cells and from RPTP μ -transfected 3T3 cells. In those studies, we failed to detect any RPTP μ -associated proteins in the 120–130-kD

or 90–100-kD region, where cadherins and catenins should migrate (Gebbinck et al., 1995; Zondag, G.C., and M.F. Gebbinck, 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 RPTP μ and associating proteins. Therefore, we tested four other monoclonal antibodies recognizing different epitopes in the RPTP μ 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 RPTP μ (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 RPTP μ immunoprecipitates probed with anti-RPTP μ monoclonal antibody 3G4. As can be seen from lanes 2 to 6, all anti-RPTP μ monoclonals precipitate a 200-kD protein corresponding to full-length RPTP μ and the 100-kD cleaved form of RPTP μ (Gebbinck et al., 1995). In contrast, no RPTP μ 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 1; 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 RPTP μ immunoprecipitations by various monoclonal antibodies (lanes 2–6), it is seen that only the BK2 antibody is able to precipitate cadherins. None of the other anti-RPTP μ monoclonals coprecipitates any cadherins, although they all recognize a different epitope on RPTP μ . This makes it very unlikely that the antibodies used would interfere with a putative RPTP μ -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 p120^{cas}, a phosphotyrosine substrate of tyrosine kinases (Reynolds et al., 1994; Shibamoto et al., 1995). To further examine the putative interaction between RPTP μ and cadherin complexes, we probed cadherin/catenin immunoprecipitates with anti-RPTP μ antibody and vice versa. As shown in Fig. 1 B, no RPTP μ is detectable in cadherin complexes immunoprecipitated with antibodies against cadherin, α - and β -catenin, or p120^{cas}. Control experiments show that these antibodies do precipitate their respective antigens, and that antibodies to α -catenin, β -catenin, and p120^{cas} do coprecipitate cadherins. Conversely, no cadherin or catenin/p120^{cas} proteins are detectable in anti-RPTP μ immunoprecipitates (Fig. 1 B; see also Fig. 1 A). These results reinforce the notion that there is no physiological interaction between RPTP μ and cadherin complexes.

Overexpression of RPTP μ Does Not Induce Cadherin Association

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

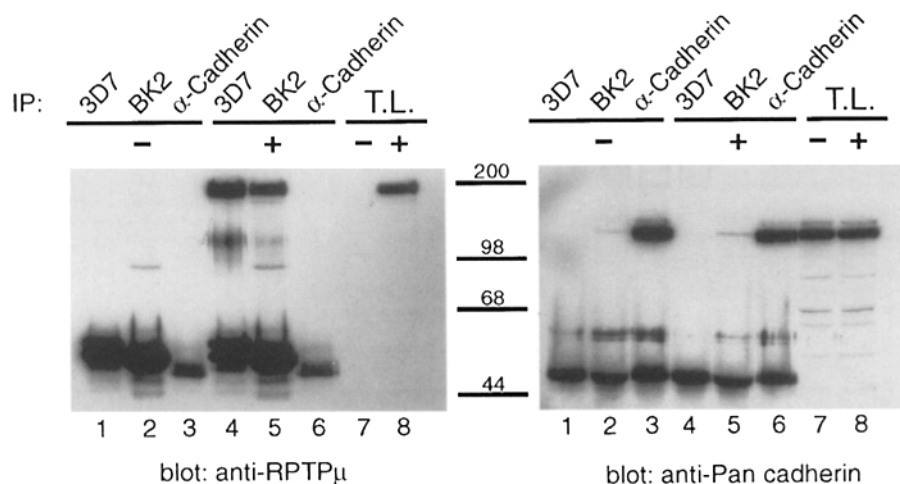


Figure 2. Overexpression of RPTP μ does not result in association with endogenous cadherins. COS cells were transfected with either empty vector (lanes 1, 2, 3, and 7, as indicated by a minus) or with RPTP μ cDNA (lanes 4, 5, 6, and 8, as indicated by a plus) and lysed after 2 d. Cell lysates were analyzed for RPTP μ /cadherin complexes by immunoprecipitation using the anti-RPTP μ antibodies BK2 and 3D7, as well as an anti-Pan cadherin monoclonal antibody. Total cell lysates and immunoprecipitates were analyzed by immunoblotting using anti-RPTP μ antibody 3G4 (left panel), or anti-Pan cadherin antibody (right panel). It is seen that 3D7 does not coprecipitate any cadherins nor that the anti-cadherin antibody coprecipitates any RPTP μ . In contrast, the BK2 antibody precipitates cadherin from both empty vector and RPTP μ transfected cells (right panel, lanes 2 and 5).

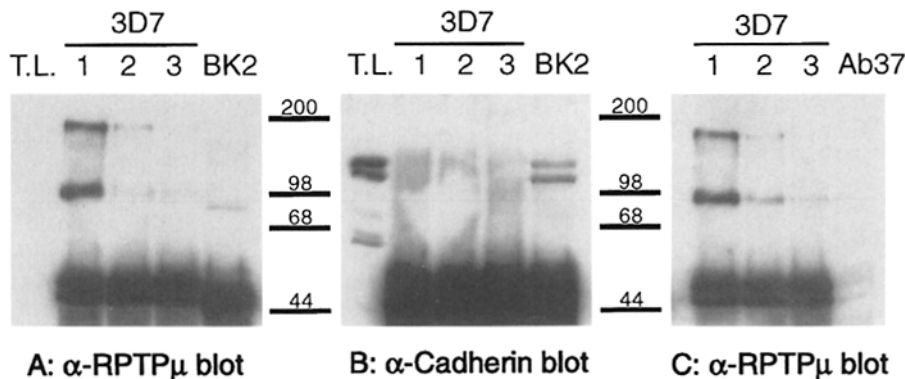


Figure 3. Monoclonal antibody BK2 precipitates cadherins independently of RPTP μ . Mv1Lu cell lysate was depleted from RPTP μ by three consecutive immunoprecipitations using antibody 3D7 (lanes 1, 2 and 3, respectively). Half of the depleted lysate was subsequently analyzed by immunoprecipitation using the BK2 antibody. In *A*, total lysate and immunoprecipitations are probed with anti-RPTP μ antibody 3G4. Note that no RPTP μ 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 RPTP μ . (*C*) No residual RPTP μ is present after three consecutive 3D7-immunoprecipitations. Half of the RPTP μ depleted cell lysate as used above was supplemented with an equal volume of 2 \times BUSS buffer. Polyclonal antibody 37, raised against a COOH-terminal RPTP μ peptide, was then used to precipitate any residual RPTP μ which may have been unrecognized by the 3D7 antibody. No RPTP μ is detectable in the Ab 37 precipitate, confirming complete RPTP μ depletion of the mink cell lysates.

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

Nonspecific Precipitation of Cadherins by BK2

To further examine the observed reactivity of BK2 against cadherins, we depleted RPTP μ from Mv1Lu 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-RPTP μ antibody. It is seen that a single 3D7 precipitation brings down \sim 90% of all RPTP μ from the lysate; after two subsequent precipitations, virtually no RPTP μ can be precipitated anymore. As expected, subsequent precipitation with BK2 antibody does not bring down any RPTP μ .

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 RPTP μ -depleted lysates, consistent with BK2 acting nonspecifically. As an additional control, we used polyclonal antiserum 37 raised against the RPTP μ COOH terminus, which recognizes all forms of RPTP μ (i.e., cleaved, uncleaved, glycosylated, and nonglycosylated forms; Gebbink et al., 1995). As shown in Fig. 3 *C*, antibody 37 does not precipitate any RPTP μ from the 3D7-precleared lysates, demonstrating that RPTP μ depletion was complete. This indicates that the BK2 antibody directly recognizes an epitope on cadherins and that it precipitates cadherins independently of RPTP μ .

Concluding Remarks

In the present study, we have examined the putative association between RPTP μ and cadherins. We found no evidence for such an interaction. In particular, our experiments reveal that the reported RPTP μ /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-RPTP μ . Using recombinant baculovirus, we have generated native fusion proteins consisting of GST fused to the complete intracellular domain of RPTP μ . 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 transferred 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 RPTP μ 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 RPTP μ does not associate with cadherins or with catenins is supported by preliminary analysis of RPTP μ knockout mice (Gebbink, M.F., E. Feiken, G.C. Zondag, and W.H. Moolenaar, manuscript in preparation). In these RPTP μ -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 RPTP μ thus remains a challenge for future studies.

We thank Drs. N. Tonks, P. Bringuier, J. Schalke, R. Kypta, A. Reynolds, O. Huber, and R. Kemler for providing antibodies, and Drs. J. Neeffjes and R. Kypta for helpful discussions.

This work was supported by the Dutch Cancer Society.

Received for publication 26 April 1996 and in revised form 15 July 1996.

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