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.
tained contradict the earlier findings by Brady-Kalnay et al. (1995). Our data indicate that RPTPα does not interact with cadherins or catenins and 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/α) and 3D7 (IgG2a/α) directed against the RPTPα ectodomain has been described (Gebbink et al., 1995). Monoclonal antibodies 1D5 (IgG1/α) and B21 (IgG2a/α) 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 RPTPα 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 p120cat 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-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 μg/ml DEAE-dextran and 5 μg GMt2-hFLα 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% FCS and antibiotics. The generation and characterization of monoclonal antibodies 1E1 (isotype IgG1/κ) and 3D7 (IgG2a/κ) 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 p120cat 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).

**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 μg/ml Pefabloc (Fluka Chemie AG, Switzerland), 200 μM phenylarsenoxide, 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-lina) 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× 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× BUSS buffer. Total lystate was prepared by adding 4× 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 3% nonfat dry milk in TBST (20 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 RPTPα Immunoprecipitates**

To examine whether RPTPα and cadherins may physically interact, we used various antibodies in immunoprecipitation assays. In previous experiments, we used monoclonal antibody 3D7 to precipitate RPTPα from surface-iodinated or metabolically labeled Mv1Lu cells and from RPTPα-transfected 3T3 cells. In those studies, we failed to detect any RPTPα-associated proteins in the 120–130-kD region.
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. 1A (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. 1A, 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 RPTPµ 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-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 phosphorylserine 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 cadherin/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...
3D7 immunoprecipitate lacks detectable cadherin. Surprisingly, however, the BK2 antibody precipitates small amounts of cadherin from both RPTPμ-deficient control cells and RPTPμ-overexpressing cells. It thus appears that the BK2 antibody acts in a nonspecific manner, as it precipitates cadherins independently of RPTPμ. These results show that, even after high overexpression, RPTPμ fails to associate with cadherins.

**Nonspecific Precipitation of Cadherins by BK2**

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

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

**Concluding Remarks**

In the present study, we have examined the putative association between RPTPμ and cadherins. We found no evidence for such an interaction. In particular, our experiments reveal that the reported RPTPμ/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-RPTPμ. Using recombinant baculovirus, we have generated native fusion proteins consisting of GST fused to the complete intracellular domain of RPTPμ. 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 RPTPμ fusion proteins in bacteria, which is likely to yield misfolded or denaturated protein. Moreover, the bacterial products were applied in rather large amounts, with a high risk of nonspecific protein–protein interactions.

That RPTPμ does not associate with cadherins or with catenins is supported by preliminary analysis of RPTPμ knockout mice (Gebbink, M.F., E. Feikens, G.C. Zondag, and W.H. Moolenaar, manuscript in preparation). In these RPTPμ-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 RPTPμ thus remains a challenge for future studies.

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**References**


