Dynamic Interaction of PTPμ with Multiple Cadherins In Vivo

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Abstract. There is a growing body of evidence to imply that reversible tyrosine phosphorylation, as an important mechanism in the control of the adhesive function of cadherins. We previously demonstrated that the receptor protein tyrosine phosphatase PTPμ associates with the cadherin–catenin complex in various tissues and cells and, therefore, may be a component of such a regulatory mechanism (Brady-Kalnay, S.M., D.L. Rimm, and N.K. Tonks. 1995. J. Cell Biol. 130:977–986). In this study, we present further characterization of this interaction using a variety of systems. We observed that PTPμ interacted with N-cadherin, E-cadherin, and cadherin-4 (also called R-cadherin) in extracts of rat lung. We observed direct interaction between PTPμ and E-cadherin after coexpression in Sf9 cells. In WC5 cells, which express a temperature-sensitive mutant form of v-Src, the complex between PTPμ and E-cadherin was dynamic, and conditions that resulted in tyrosine phosphorylation of E-cadherin were associated with dissociation of PTPμ from the complex. Furthermore, we have demonstrated that the COOH-terminal 38 residues of the cytoplasmic segment of E-cadherin was required for association with PTPμ in WC5 cells. Zondag et al. (Zondag, G., W. Moolenaar, and M. Gebbink. 1996. J. Cell Biol. 134:1513–1517) have asserted that the association we observed between PTPμ and the cadherin–catenin complex in immunoprecipitates of the phosphatase arises from nonspecific cross-reactivity between BK2, our antibody to PTPμ, and cadherins. In this study we have confirmed our initial observation and demonstrated the presence of cadherin in immunoprecipitates of PTPμ obtained with three antibodies that recognize distinct epitopes in the phosphatase. In addition, we have demonstrated directly that the anti-PTPμ antibody BK2 that we used initially did not cross-react with cadherin. Our data reinforce the observation of an interaction between PTPμ and E-cadherin in vitro and in vivo, further emphasizing the potential importance of reversible tyrosine phosphorylation in regulating cadherin function.

The cadherins are a major family of calcium-dependent, homophilic cell adhesion molecules that are concentrated at specialized contact points in the cell termed adherens junctions (for review see Gumbiner, 1996). The cadherins are transmembrane proteins that possess an extracellular segment, characterized by the presence of calcium-binding motifs, and an intracellular segment that is highly conserved between members of the family (for review see Takeichi, 1995). The intracellular segment serves as the site of interaction with proteins termed catenins (α-, β-, and γ-catenin) (for review see Gumbiner, 1995). It appears that β-catenin and γ-catenin/plakoglobin, which are related to the product of the segment polarity gene armadillo, bind directly to the cytoplasmic segment of cadherin, whereas α-catenin, which is related to the cytoskeleton-associated protein vinculin, binds to β/γ catenin and functions to link the complex to the actin cytoskeleton (for review see Gumbiner, 1995). The intracellular, catenin-binding segment of the cadherins is essential for adhesion; mutations in this segment can disrupt adhesion even in the presence of an intact extracellular segment (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989). Thus, cadherin-mediated adhesion requires the intact cadherin–catenin complex and association with the actin cytoskeleton.

Mutations have been detected in components of the cadherin–catenin complex in several tumors, and destabilization of cadherin-mediated adhesion has been linked with invasion and malignant progression (for reviews see Birchmeier and Behrens, 1994, Birchmeier, 1995). In addi-
tion, the junctions in normal cells are dynamic and tyrosine phosphorylated rapidly and reversibly (Volberg et al., 1991). There is now a growing body of evidence to link the loss of adhesive function and the destabilization of adherens junctions with changes in the state of phosphorylation of tyrosyl residues in components of the cadherin–catenin complex (for reviews see Birchmeier and Behrens, 1994; Brady-Kalnay and Tonks, 1995). Expression of the protein tyrosine kinase (PTK) v-Src causes aberrant tyrosine phosphorylation that results in disruption of adherens junctions, in the absence of an effect on desmosomes and tight junctions (Warren and Nelson, 1987). Similarly, treatment of MDCK cells with vanadate, a broad specificity inhibitor of members of the protein tyrosine phosphatase (PTP) family of enzymes, results in tyrosine phosphorylation of proteins at adherens junctions and the deterioration of junctional structures (Volberg et al., 1992). Furthermore, β-catenin was observed to be heavily phosphorylated on tyrosyl residues in rat fibroblasts transformed by v-Src, coincident with changes in cell–cell aggregation (Matsuyoshi et al., 1992). In addition, these effects were abrogated by the PTK inhibitor herbimycin A and promoted by the PTP inhibitor vanadate. Interestingly, a temperature-sensitive mutant of v-Src destabilized cadherin-dependent adhesion at the permissive temperature, coincident with tyrosine phosphorylation of E-cadherin, β-catenin, or cytoskeletal components (Behrens et al., 1993; Takeda et al., 1995). The EGF receptor and Met, the receptor for scatter factor, phosphorylate components of the cadherin–catenin complex, and the EGF receptor has been observed to bind directly to β-catenin and to associate with the cadherin–catenin complex in epithelial cells (Hoschuetzky et al., 1994; Ochiai et al., 1994; Shibamoto et al., 1994). These observations suggest that the integrity of adherens junctions is regulated in part at the level of reversible tyrosine phosphorylation that results from the coordinated and competing actions of PTKs and PTPs. Therefore, a prerequisite to understanding fully the significance of tyrosine phosphorylation in the control of cadherin–catenin function will be the identification and characterization of specific PTKs and PTPs that associate with and modify the phosphorylation status of these proteins.

In examining the physiological significance of the tyrosine phosphorylation of the cadherin–catenin complex, we have obtained data that implicate the receptor PTP, PTPα, as a potential regulator of this complex. PTPα is characterized by an extracellular segment that contains one MAM (Meprin/A5/PTPα) domain, one immunoglobulin domain, and four fibronectin type III repeats (Gebbink et al., 1991). This combination of motifs suggested that PTPα may function in cell–cell adhesion. In fact, we (Brady-Kalnay et al., 1993) and others (Gebbink et al., 1993; Sap et al., 1994) demonstrated that PTPα, and the structurally related PTPκ, participate in homophilic binding interactions. Ectopic expression of recombinant PTPα in Sf9 cells induces aggregation of these normally nonadhesive cells (Brady-Kalnay et al., 1993; Gebbink et al., 1993). Subsequently, we determined that the homophilic binding site within the extracellular segment of PTPα resides in the immunoglobulin domain (Brady-Kalnay and Tonks, 1994). In addition, it has been shown that the MAM domain plays a role in cell–cell aggregation possibly by “sorting” of PTPα from closely related molecules, such as PTPκ, during cell aggregation (Zondag et al., 1995). More recent data suggest that one aspect of PTPα function in vivo may be to affect cell adhesion by regulating the adhesive properties of the cadherin–catenin complex.

We observed that in the MvLu lung cell line, which expresses PTPα, catenins, and cadherins endogenously, immunoprecipitates of PTPα contained cadherins, α-catenin, and β-catenin (Brady-Kalnay et al., 1995). In fact, at least 80% of the total cellular cadherins appeared to be associated with PTPα in MvLu cells. Similarly, complexes between PTPα and cadherins were detected in rat heart, lung, and brain tissues, where PTPα is expressed at high levels (Brady-Kalnay et al., 1995). The results of binding studies in vitro suggest that this association results from a direct interaction between the intracellular segment of PTPα and the intracellular domain of E-cadherin (Brady-Kalnay et al., 1995). Our results raised the possibility that a component of the cadherin–catenin complex may be an endogenous substrate for PTPα. Subsequently, several other laboratories have reported the observation of interactions between cadherin–catenin complexes and both receptor and nontransmembrane PTPs in a variety of cell systems (Balsamo et al., 1996; Fuchs et al., 1996; Kyppta et al., 1996; Aicher et al., 1997; Cheng et al., 1997).

In this paper, we report the results of a further characterization of the association between PTPα and cadherin–catenin complexes. We have identified the cadherins that associate with PTPα in vivo from lysates of rat lung as N-cadherin, E-cadherin, and cadherin-4 (also called R-cadherin). We have used a number of systems to characterize further the association of PTPα and E-cadherin and have demonstrated that the COOH-terminal 38 residues of the cytoplasmic segment of E-cadherin is necessary for binding of PTPα. Furthermore, we have shown that conditions that result in tyrosine phosphorylation of E-cadherin also result in dissociation of PTPα from the complex. A recent article from Zondag et al. (1996) argues that PTPα does not associate with cadherins and suggests that our observations are the result of nonspecific cross-reactivity between BK2, our antipeptide antibody to PTPα, and cadherins. We present several lines of data to substantiate the validity of our original observation of the association between PTPα and the cadherin–catenin complex and to refute the assertion of Zondag et al. that our observation arises from nonspecific antibody cross-reactivity.

Materials and Methods

Expression Vectors and Cell Lines

Expression vectors for wild-type and mutant murine E-cadherins were either generously provided by Masatoshi Takeichi (Kyoto University, Japan) (Nagafuchi and Takeichi, 1988; Nose et al., 1988) or described in Chen et al. (1997). WC5 cells were derived from neonatal rat cerebellar cells by transformation with a mutant of Rous sarcoma virus (LA90) that is temperature-sensitive for transformation (Giotta and Cohn, 1981). At the permissive temperature, 33°C, v-Src is active as a PTK, and the cells are transformed. At 39°C, the v-Src PTK is relatively inactive, and the cells display an epithelial morphology. WC5 cells were maintained at 33°C in DME (GIBCO BRL, Gaithersburg, MD) containing 10% fetal bovine se-

1. Abbreviations used in this paper: GST, glutathione S transferase; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase.
rum and 10 μg/ml gentamicin. The isolation of stable cell lines expressing full-length and deletion constructs of E-cadherin has been described (Chen et al., 1997). At least two clonal cell lines for each E-cadherin deletion mutant were tested in each of the experiments described. MvLu cells were cultured as described previously (Brady-Kalnay et al., 1995). The cell line MCF10AneoN was generously provided by Bonnie Sloan (Wayne State University, Detroit, MI) and cultured as described in Kinch et al. (1997). S9 cells (CRL 1711; American Type Culture Collection, Rockville, MD) were maintained at 27°C in Grace's Insect Medium Supplemented (GIBCO BRL) containing 10% fetal bovine serum and 10 μg/ml gentamicin.

Expression in Sf9 Cells

The following recombinant baculoviruses were used: (a) expressing full-length PTPα, described previously (Brady-Kalnay et al., 1993); (b) expressing E-cadherin, generated using the BaculoGold Transfection System (Invitrogen Corp., Carlsbad, CA) after ligation of a full-length 2.7-kb cDNA (recovered by restriction digestion of the pBATEM2 plasmid, generously provided by M. Takeichi) into pVL1392 (Invitrogen Corp.); and (c) expressing β-catenin, generously provided by R. Krypa (University of California, San Francisco, CA). S9 cells were infected with the recombinant baculoviruses either singly or in pairwise combinations as previously described (Brady-Kalnay et al., 1993). Cells were harvested 48 h after infection by centrifugation at 3,000 g for 5 min and processed for immunoprecipitation and immunoblotting as described below.

Antibodies

Hybridoma cells expressing a rat monoclonal antibody against the extra-cellular domain of E-cadherin, ECCD-2 (Shiroyashi et al., 1986), were generously provided by Masatoshi Takeichi. Conditioned medium from these hybridomas was used in experiments. A mouse monoclonal antibody to E-cadherin, antibodies to β-catenin, and antiphosphotyrosine antibody (PY20) were purchased from Transduction Labs (Lexington, KY). In the course of our experiments involving antibody recognition of E-cadherin fusion proteins, we determined that the anti-E–cadherin antibody from Transduction Labs recognized the juxtamembrane half of the intracellular segment. Pan-cadherin antibodies (monoclonal and polyclonal), which react with the conserved COOH-terminal 24 amino acids of the cadherin cytoplasmic segment, were purchased from Sigma Chemical Co. (St. Louis, MO). The cadherin-4 antibody (120A) was generously provided by ICOS Corp. (Seattle, WA). Antibody to N-cadherin (BD7873) was generously provided by Dr. J. Hemperly at Becton Dickinson Labs (Res. Triangle Park, NC) and has been described previously (Payne et al., 1996). Monoclonal antibodies to the intracellular segment of PTPα (SK series) and monoclonal antibody BK-2, generated against a peptide derived from the extracellular segment of PTPα, have been described previously (Brady-Kalnay et al., 1993; Brady-Kalnay and Tonks 1994). Polyclonal antibodies to glutathione S transferase (GST) (Brady-Kalnay et al., 1993) and the FG6 monoclonal antibody to PTP1B (Flint et al., 1993) have been described previously.

Binding Assays In Vitro

The GST fusion protein of the extracellular segment of PTPα (EXTRA-PTPα) has been described previously (Brady-Kalnay et al., 1993). Two GST-E-cadherin fusion proteins were generated that contain either amino acids 572–631 (the juxtamembrane-half of the cytoplasmic segment, JM E-cad) or amino acids 648–729 (the COOH-terminal, cadherin-binding portion of the intracellular segment, CB E-cad). Proteins were expressed in Escherichia coli and purified using glutathione Sepharose (Brady-Kalnay et al., 1995). In slot blot analyses, purified protein samples were adsorbed to nitrocellulose, rinsed in a solution that contained the bead apparatus (Bio-Rad Laboratories, Hercules, CA). The nitrocellulose strip was blocked in 5% nonfat dry milk in TTBS (20 mM Tris, pH 7.5, 660 mM NaCl, 0.05% Tween-20) and then incubated with primary antibody for 16 h at 4°C. The blot was washed in TTBS and then developed using horseradish peroxidase–conjugated secondary antibodies and Enhanced Chemiluminescence reagents (Amersham Corp., Arlington Heights, IL).

Preparation of Samples for Immunoprecipitation

Triton-soluble lysates of rat lung and MvLu cells were prepared as described (Brady-Kalnay et al., 1995). WCs cells were lysed in 20 mM Tris, pH 7.5, 2 mM CaCl2, 1% Triton X-100, 5 μg/ml leupeptin, 5 μg/ml apro-
Figure 1. Coimmunoprecipitation of PTPµ and cadherin from lysates of MvLu cells. Lysates of MvLu cells were subjected to immunoprecipitation with two different concentrations of anti-PTPµ antibody BK2 or an isotype-matched antibody to PTP1B (FG6), as well as the pan-cadherin and anti-β-catenin antibody. The relative amounts of antibody heavy chain in each immunoprecipitate are shown in a Ponceau S stain of the immunoblot (A). Immunoblots using pan-cadherin antibody were performed on the immunoprecipitates (B). The quantity of cadherin remaining in the supernatant after immunoprecipitation was assessed by immunoblotting (C). The results illustrate that the majority of cadherin in the lysate coimmunoprecipitated with PTPµ.

Reconstitution of the Interaction between PTPµ and E-cadherin in Sf9 Cells

A major contention of the paper by Zondag et al. (1996) was that the association we observed between PTPµ and cadherin was an artifact arising from nonspecific cross-reactivity between the anti-PTPµ antibody, BK2, and cadherins. We have now addressed this issue in a variety of systems.

We have reconstituted the complex in Sf9 cells by expression of the individual components using recombinant baculoviruses. PTPµ, E-cadherin, and β-catenin were expressed singly or in pairwise combinations, and expression was verified by immunoblotting cell lysates with the appropriate antibodies (Fig. 3, Lysate). E-cadherin was recovered in anti-PTPµ immunoprecipitates, prepared using the BK2 antibody, only from cells in which both proteins were coexpressed (Fig. 3, BK2 IPs, E-cadherin blots, lane 4). The BK2 antibody did not precipitate E-cadherin from cell lysates in the absence of PTPµ, thus ruling out the possibility that the antibody recognized E-cadherin nonspecifically (Fig. 3, BK2 IPs, E-cadherin blots, lane 2). Similar observations were made when we examined immunoprecipitates with the anti-PTPµ antibody BK2 from lysates of MCF10A cells, which express large amounts of E-cadherin but do not express detectable levels of PTPµ. Even in the presence of substantial quantities of E-cadherin and the inclusion of large quantities of the antibody, BK2 did not precipitate E-cadherin from lysates of MCF10A cells (data not shown). When the experiment was repeated using a distinct antibody to PTPµ, SK18, which recognizes an epitope in the intracellular portion of the enzyme, the same observation was made. E-cadherin was recovered in anti-PTPµ immunoprecipitates only from lysates of cells coexpressing the phosphatase together with E-cadherin and not cells expressing E-cadherin alone (Fig. 3, SK18 IPs). Furthermore, the formation of a complex was also revealed by the reverse immunoprecipitation/blotting strategy, in that PTPµ was recovered in immunoprecipitates of E-cadherin, but only from lysates of cells in which both proteins were expressed (Fig. 3, Ecad IPs, PTPµ blots, lane 4).

The data also illustrate that PTPµ did not interact with β-catenin in this system (see Fig. 3, anti-β-catenin blots of BK2 and SK18 immunoprecipitates and anti-PTPµ blots of β-catenin immunoprecipitates), whereas β-catenin was recovered in immunoprecipitates of E-cadherin (Fig. 3, Ecad IPs and β cat IPs), indicating that the protein was produced in a conformation appropriate for complex formation. This observation is consistent with our previous results from blot-overlay assays, which revealed a direct interaction between PTPµ and E-cadherin in vitro (Brady-Kalnay et al., 1995) and indicates that the binding of PTPµ to E-cadherin is not mediated by β-catenin.

These data reveal that PTPµ/E-cadherin complexes are recovered by immunoprecipitation with two distinct antibodies to the phosphatase or with antibody to E-cadherin. In addition, anti-PTPµ antibodies did not precipitate E-cad-
herin in the absence of the phosphatase. Therefore, it is highly unlikely that this result could be explained by nonspecific antibody cross-reactivity.

**The PTPµ/E-cadherin Complex Is Dynamic In Vivo**

WC5 is a Rous sarcoma virus–transformed, rat cerebellar cell line that expresses a temperature-sensitive mutant of the v-Src PTK. When grown at the nonpermissive temperature (39°C), the Src PTK displays little activity, and the cells manifest properties of astrocytes (Giotta and Cohn, 1981). In contrast, when grown at the permissive temperature (33°C), Src is active and the cells are transformed. The expression of endogenous PTPµ, both the unprocessed (200 kD) and proteolytically processed (100 kD) forms of the enzyme, and ectopically expressed E-cadherin was not affected by switching between permissive and nonpermissive temperatures (Fig. 4 A).

We used the WC5 cell line that expresses full-length E-cadherin to assess the effect of tyrosine phosphorylation on the PTPµ/cadherin complex by comparing the extent to which E-cadherin coimmunoprecipitated with PTPµ at the permissive and nonpermissive temperatures. As shown in Fig. 4, PTPµ antibodies coimmunoprecipitated E-cadherin well at the nonpermissive temperature (39°C) but poorly at the permissive temperature (33°C) for the Src PTK (Fig. 4 B), despite the fact that expression of E-cadherin was apparently unaltered at 39°C compared with 33°C (Fig. 4 A). Interestingly, E-cadherin was immunoprecipitated with antiphosphotyrosine antibodies at 33°C but to a lesser extent at 39°C. Under harsh detergent conditions (RIPA buffer), we observed that E-cadherin was tyrosine phosphorylated directly at the permissive temperature (data not shown). Therefore, our data indicate an inverse correlation between the presence of PTPµ in the cadherin–catenin complex and the phosphorylation of tyrosyl residues in E-cadherin. The fact that a complex between PTPµ and E-cadherin was detected at 39°C but not at 33°C, using identical conditions for cell lysis and immunoprecipitation at each temperature, indicates that the complex we observe is dynamic. Furthermore, although the potential existence of a phosphorylation-sensitive, cross-reacting epitope is not formally excluded, these data also indicate that it is unlikely that the complex we detect can be explained by nonspecific antibody cross-reactivity.

**Identification of the Catenin-binding Domain within the Intracellular Segment of E-cadherin as the Site of Interaction with PTPµ**

In our previous study, we demonstrated that E-cadherin interacts with PTPµ both in vitro and in vivo. Therefore,
we set out to identify the binding site for PTP\(\mu\) in E-cadherin. In our initial report, we demonstrated that the intracellular segment of PTP\(\mu\) interacted directly with the intracellular segment of E-cadherin (Brady-Kalnay et al., 1995). To determine more precisely the location of the PTP\(\mu\)-binding site within the cytoplasmic segment of E-cadherin, we used the WC5 cells, which express PTP\(\mu\) endogenously but do not express endogenous E-cadherin. We used a series of WC5 cell lines that express ectopically various forms of E-cadherin containing deletions in the cytoplasmic segment. We tested for the effects of the deletions in E-cadherin on its ability to associate with PTP\(\mu\) in a cellular context by immunoprecipitating PTP\(\mu\) from the various WC5 cell lysates and determining whether E-cadherin coimmunoprecipitated with the phosphatase. As shown in Fig. 5 A, WC5 cell lines expressing five deletion mutants of E-cadherin (Chen et al., 1997) were used. Specifically, these included (a) the full-length E-cadherin molecule as a control (Ecad), (b) a control from which the entire cytoplasmic segment of E-cadherin was deleted (CDD), (c) a mutant in which the COOH-terminal 38 residues were deleted (CB), (d) a mutant in which the internal domain of the intracellular segment was deleted (ID), and (e) a mutant in which the juxtamembrane domain of E-cadherin was deleted (JM).

The WC5 cell lines expressed each of the E-cadherin deletion mutants to similar levels. Each of the mutants was precipitated with antibodies to the extracellular segment of E-cadherin and migrated at the expected molecular weight (Fig. 5 B). A monoclonal antibody to the cytosolic PTP, PTP1B (Flint et al., 1993), was used as a negative control, and although capable of immunoprecipitating PTP1B (data not shown), it did not immunoprecipitate E-cadherin (Fig. 5 C). Using the BK2 antibody to PTP\(\mu\), which recognizes a peptide sequence in the MAM domain within the extracellular segment of PTP\(\mu\) (Brady-Kalnay and Tonks, 1994), we observed that E-cadherin mutants bearing a deletion of the COOH-terminal 38 amino acids did not associate with PTP\(\mu\) (Fig. 5 D). Thus, PTP\(\mu\) failed to coimmunoprecipitate E-cadherin from two distinct WC5 lines expressing such E-cadherin deletion mutants (CB1 and CB2) and from a line expressing E-cadherin from which the entire cytoplasmic segment was deleted (CDD). These results indicate that the COOH-terminal 38 residues of E-cadherin is required for the interaction with PTP\(\mu\).

Originally, our observation of association between PTP\(\mu\) and cadherin was founded primarily upon experiments performed with one antibody to the phosphatase, designated BK2, which recognized an epitope in the extracellular segment of the enzyme. We generated previously the SK series of monoclonal antibodies to the intracellular domain of PTP\(\mu\) (Brady-Kalnay et al., 1993). By using histidine-tagged fusion proteins comprising various portions of the intracellular segment of PTP\(\mu\), we identified two classes of SK antibodies. One class recognized epitopes in the juxtamembrane segment of the phosphatase whereas the other recognized epitopes in the first phosphatase domain (data not shown). To extend the scope of our analysis of the association of PTP\(\mu\) and cadherins, we tested whether the SK series monoclonal antibodies were able to immunoprecipitate the PTP\(\mu\)-E-cadherin complex from the various WC5 cell lines, concentrating on one with an epitope in the juxtamembrane segment of PTP\(\mu\) (SK7) and another that recognized the first PTP domain (SK18). As shown in Fig. 5, E and F, these antibodies, like BK2, immunoprecipitated the PTP\(\mu\)/E-cadherin complex, but only from cells expressing forms of E-cadherin in which the COOH-terminal 38 residues were present. Similar data were obtained with all SK series antibodies tested (data not shown).

**The Anti-PTP\(\mu\) Antibody, BK2, Does Not Cross-react with Cadherins**

The BK2 antibody was generated against a peptide derived from the NH\(_2\) terminus of human PTP\(\mu\) (Brady-Kalnay et al., 1994) and did not show cross-reactivity with the PTP\(\mu\)-like enzymes PTP\(\xi\) and PTP/PCP-2. The peptide sequence displayed no obvious similarity to the intracellular segment of cadherin, which contains the site of interaction with PTP\(\mu\) (Brady-Kalnay et al., 1995 and Fig. 5). Despite the lack of obvious sequence similarity between this peptide and the cadherins, we addressed the issue of cross-reactivity further in the following experiment.

We examined whether the anti-PTP\(\mu\) antibody BK2 recognized a GST–E-cadherin fusion protein, purified after expression in E. coli, in a direct binding assay under non-denaturing conditions in vitro. Our original study demonstrated an interaction between the intracellular segment of PTP\(\mu\) and the intracellular segment of E-cadherin (Brady-Kalnay et al., 1995). Furthermore, the data presented here in Fig. 5 show that the COOH-terminal 38 residues of E-cadherin were required for association with PTP\(\mu\). Therefore, any potential cross-reacting epitopes would be in this segment. We tested whether the BK2 antibody binds to the intracellular segment of E-cadherin under non-denaturing conditions. We used the following four GST fusion proteins: (a) GST alone, (b) the juxtamembrane half of the intracellular segment of E-cadherin (JM E-cad), (c) the COOH-terminal, catenin-binding portion.
of the intracellular segment of E-cadherin (CB E-cad), and (d) the extracellular domain of PTPm (EXTRA PTPm). An antibody to GST recognized all of the fusion proteins (Fig. 6A). One of the commercially available antibodies reacted with the juxtamembrane half of E-cadherin (Fig. 6B), whereas the polyclonal, pan-cadherin antibody reacted with the catenin-binding half of the E-cadherin intracellular segment (Fig. 6C). The SK15 antibody, to the intracellular segment of PTPm, did not recognize any of the fusion proteins (Fig. 6D), whereas antibody BK2 interacted only with the extracellular segment of PTPm (Fig. 6E). Thus, under these conditions, the BK2 antibody did not recognize the intracellular segment of E-cadherin, which contains the binding site for PTPm (Fig. 5), even when 10 μg of the purified protein was applied to the nitrocellulose filter to ensure maximal binding of the protein to the nitrocellulose, and the blot was overexposed. These data provide further indication that the BK2 antibody does not recognize E-cadherin nonspecifically.

Discussion
Components of adherens junctions are subjected to rapid, reversible tyrosine phosphorylation in a cellular context (Volberg et al., 1991). Tyrosine phosphorylation of the cadherin–catenin complex has been observed under a variety of conditions, including in response to oncprotein PTKs, such as Src (Matsuyoshi et al., 1992; Behrens et al., 1993), or to oncogenic forms of Ras (Kinch et al., 1995) and following stimulation of receptor PTKs, such as EGF receptor and Met (Shibamoto et al., 1994). In addition, PTKs such as EGF receptor and c-erbB2 have been observed to associate with the cadherin–catenin complex in vivo (Hoschuetzky et al., 1994; Ochiai et al., 1994). The reversibility of tyrosine phosphorylation in vivo depends upon the coordinated action of both PTKs and PTPs. Therefore, to understand fully the regulation of cadherin function by reversible tyrosine phosphorylation, it will be necessary to identify and characterize the phosphatases that act upon adhesion complexes in vivo. Our observation of association between a receptor PTP, PTPm, and cadherins in various tissues and cells is consistent with a role for this phosphatase in regulating cadherin function and lends further support to the regulatory importance of tyrosine phosphorylation in cell adhesion.

In this study, we have demonstrated that PTPm inter-
acted with N-cadherin, E-cadherin, and cadherin-4 (also called R-cadherin) in extracts of rat lung. Although PTP\(\text{m}\) can interact with several cadherins, it displays a restricted tissue distribution. Therefore, one would anticipate that, if regulation of cadherin function by reversible tyrosine phosphorylation was a general phenomenon, there would be additional PTPs that function in a manner analogous to PTP\(\text{m}\) in other cell types. Subsequent to our original demonstration of association between PTP\(\text{m}\) and the cadherin–catenin complex (Brady-Kalnay et al., 1995), several reports have appeared that substantiate the general principle that members of the PTP family may be important regulators of cadherin-mediated adhesion. Association of a number of receptor and nontransmembrane PTPs with different members of the cadherin family in a variety of cell systems has now been reported. PTP\(\kappa\), a receptor PTP that is closely related in structure to PTP\(\text{m}\) (\(\sim 75\%\) sequence identity with the same overall arrangement of structural motifs), has been shown to associate directly with \(\beta\)-catenin and plakoglobin (Fuchs et al., 1996). Interestingly, PTP\(\kappa\) displays a much broader expression pattern than PTP\(\text{m}\) (Jiang et al., 1993) and therefore may interact with cadherin–catenin complexes in many tissues. To date, four other PTPs have been shown to interact with cadherin–catenin complexes. Most recently, LAR (Aicher et al., 1997) and a novel receptor PTP, termed PTP\(\alpha\) (a close relative of PTPs \(\mu\) and \(\kappa\); Cheng et al., 1997), were also shown to associate with \(\beta\)-catenin. These authors observed that the association with \(\beta\)-catenin, like that involving PTP\(\kappa\) (Fuchs et al., 1996), required the intracellular segment of the phosphatase (Aicher et al., 1997; Cheng et al., 1997). A LAR-like receptor PTP was found to associate with the cadherin–catenin complex in PC12 cells, and this association appears to be regulated by nerve growth factor–induced tyrosine phosphorylation of the PTP itself (Kypka et al., 1996). In addition, a PTP1B-like cytoplasmic phosphatase has been shown to interact with N-cadherin (Balsamo et al., 1996). The authors suggest that the association of the PTP with N-cadherin facilitates dephosphorylation of \(\beta\)-catenin, which is required for N-cadherin–mediated adhesion and its association with the actin cytoskeleton.

In contrast to this consensus view of the potential importance of PTPs in regulating the tyrosine phosphorylation of cadherin–catenin complexes in vivo, one report (Zondag et al., 1996) has questioned the validity of our original observation. Therefore, we will respond in detail to the various issues raised in the paper by Zondag et al., in an attempt both to resolve this controversy and clarify the various issues. Zondag et al. report the generation of antibodies to an undefined epitope(s) in the ectodomain of PTP\(\mu\) that fail to coimmunoprecipitate cadherin–catenin complexes. After successive rounds of immunoprecipitation with one of these antibodies, 3D7, to “clear” PTP\(\mu\) from the cell lysate, the authors subjected the cleared lysate to immunoprecipitation with our antibody BK2. Even though they were unable to detect PTP\(\mu\) in the cleared lysate by immunoblotting with their antibodies, they still observed cadherin in BK2 immunoprecipitates. From this, the authors concluded that the interaction we observed was due to nonspecific cross-reactivity between BK2 and cadherin. There are two problems with this experiment and the conclusion drawn from it. Firstly, the authors did not blot the cleared lysate with BK2 to check whether there was a pool of PTP\(\mu\) that was not recognized by their antibodies but was detected by BK2. Secondly, although the authors made the strong assertion of nonspecific cross-reactivity between BK2 and cadherin, they failed to demonstrate such cross-reactivity in a direct binding assay.

The following observations refute their argument. First, we used S9 cells, which do not contain detectable levels of endogenous PTP\(\mu\) or E-cadherin, to express these proteins and reconstitute the complex. Through this approach we demonstrated that E-cadherin was only recovered in immunoprecipitates of PTP\(\mu\) from lysates of cells in which both proteins had been coexpressed. Furthermore, the complex was detected in the reciprocal experiment, in which PTP\(\mu\) was recovered in immunoprecipitates of E-cadherin, but again only from lysates of cells in which both proteins had been coexpressed. Second, and importantly, BK2 did not recognize E-cadherin in a direct binding assay in vitro, using purified components under non-denaturing conditions. Third, BK2 did not immunoprecipitate E-cadherin from lysates of MCF10A cells, which do not express PTP\(\mu\) to a level that can be detected by antibody BK2 but express substantial levels of E-cadherin. The authors present data from a similar experiment in COS cells, which they state lack endogenous PTP\(\mu\). They report that cadherin is detected in BK2 immunoprecipitates whether or not PTP\(\mu\) was expressed ectopically. However, using a number of antibodies to PTP\(\mu\), including BK2, we detected expression of this protein in COS cells (data not shown), and thus coprecipitation of cadherin would not be unexpected. The reason for this discrepancy is unclear, although the authors did not test for the presence of PTP\(\mu\) in COS cell lysates by blotting with BK2. Fourth, in WC5 cells transformed by temperature-sensitive v-Src and expressing E-cadherin ectopically, immunoprecipitates of PTP\(\mu\) from lysates of cells cultured at the nonpermissive temperature contained coprecipitating cadherin, whereas

![Figure 6](Image)

**Figure 6.** The BK2 antibody does not cross-react with the intracellular segment of E-cadherin, which is the portion of E-cadherin required for association with PTP\(\mu\).
at the permissive temperature the levels of associated cadherin were reduced substantially (Fig. 4). It is unlikely that BK2 would display cross-reactivity only in lysates from one temperature condition. Finally, we have demonstrated interaction between PTP\textsubscript{\beta} and various members of the cadherin family using different antibodies that recognize at least three distinct epitopes in the phosphatase.

Zondag et al. (1996) presented several additional arguments to question the validity of the association we observed between PTP\textsubscript{\beta} and cadherin. For example, they cited their failure to detect the phosphatase in antacid-\textsubscript{\beta} immunoprecipitates as further evidence that PTP\textsubscript{\beta} and cadherin do not interact. However, it is important to note that in these experiments the authors used a pan-cadherin antibody that is directed against the COOH-terminal sequence that contains the segment of E-cadherin that is required for interaction with PTP\textsubscript{\beta}. Therefore, the absence of PTP\textsubscript{\beta} from these immunoprecipitates could easily be explained by steric hindrance introduced by antibody binding to cadherin. In addition, the authors dismiss our demonstration of direct interaction in blot-overlay binding studies as the result of production of the cadherin and PTP\textsubscript{\beta} fusion proteins in bacteria, which, they suggest, is likely to yield misfolded or denatured protein and result in a high risk of nonspecific protein–protein interactions.

However, the PTP\textsubscript{\beta} used as probe was produced in insect Sf9 cells, not bacteria, and was catalytically functional and in a high risk of nonspecific protein–protein interactions. Furthermore, we have detected both cadherin and PTP\textsubscript{\beta} in cadherin immunoprecipitates of PTP\textsubscript{\beta} (Brady-Kalnay et al., 1995). Our observations highlight further the potential importance of reversible tyrosine phosphorylation in regulating the adhesive properties of the cadherin family of cell adhesion molecules.

In summary, we believe that our data have established convincingly the existence of a complex between PTP\textsubscript{\beta} and various members of the family of cadherins in a number of different cell systems. In addition, we believe that we have presented data to refute convincingly the assertions of Zondag and colleagues (1996) that the association we observe between PTP\textsubscript{\beta} and cadherins is artifactual. Our observations highlight further the potential importance of reversible tyrosine phosphorylation in regulating the adhesive properties of the cadherin family of cell adhesion molecules.

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References


