Association between a Transmembrane Protein Tyrosine Phosphatase and the Cadherin–Catenin Complex

Robert M. Kypta, Hong Su, and Louis F. Reichardt
Department of Physiology and Howard Hughes Medical Institute, University of California, San Francisco, California 94143-0724

Abstract. Cadherins are calcium-dependent cell adhesion molecules that play fundamental roles in embryonic development, tissue morphogenesis, and cancer. A prerequisite for their function is association with the actin cytoskeleton via the catenins. Tyrosine phosphorylation of β-catenin, which correlates with a reduction in cadherin-dependent cell adhesion, may provide cells with a mechanism to regulate cadherin activity. Here we report that β-catenin immune precipitates from PC12 cells contain tyrosine phosphatase activity which dephosphorylates β-catenin in vitro. In addition, we show that a member of the leukocyte antigen-related protein (LAR)-related transmembrane tyrosine phosphatase family (LAR-PTP) associates with the cadherin–catenin complex. This association requires the amino-terminal domain of β-catenin but does not require the armadillo repeats, which mediate association with cadherins. The interaction also is detected in PC9 cells, which lack α-catenin. Thus, the association is not mediated by α-catenin or by cadherins. Interestingly, LAR-PTPs are phosphorylated on tyrosine in a TrkA-dependent manner, and their association with the cadherin–catenin complex is reduced in cells treated with NGF. We propose that changes in tyrosine phosphorylation of β-catenin mediated by TrkA and LAR-PTPs control cadherin adhesive function during processes such as neurite outgrowth.

Cadherins are calcium-dependent cell–cell adhesion molecules that are important regulators of morphogenesis (Takeichi, 1991). Their function requires association with the catenins, which link the highly conserved cadherin cytoplasmic domain to the actin-based cytoskeleton. β-Catenin plays a key regulatory role in cadherin function since it connects cadherins to α-catenin, which is believed to bind the actin cytoskeleton directly (Rimm et al., 1995) or through α-catenin (Knudsen et al., 1995). β-Catenin also associates with the tumor-suppressor gene product (APC) adenomatous polyposis coli, and, together with the related protein, plakoglobin, and Armadillo (the Drosophila form of β-catenin), is implicated in the Wnt/Wingless signaling pathway, which determines the dorsoventral axis in Xenopus and segment polarity in Drosophila (for references see Hinck et al., 1994; Kirkpatrick and Peifer, 1995).

Activation of several tyrosine kinases induces tyrosine phosphorylation of β-catenin (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993; Shibamoto et al., 1994). This phosphorylation correlates with perturbation of cadherin function in a number of cell types, as measured by decreases in cell aggregation (Matsuyoshi et al., 1992; Hamaguchi et al., 1993), increases in cell migration (Hamaguchi et al., 1993; Shibamoto et al., 1994), and loss of epithelial differentiation (Behrens et al., 1993). Inhibitors of tyrosine kinases block many of these physiological responses, while they are enhanced by an inhibitor of tyrosine phosphatases, sodium orthovanadate (Matsuyoshi et al., 1992; Hamaguchi et al., 1993). Furthermore, N-cadherin–dependent neurite outgrowth of neurons is blocked by vanadate (Bixby and Jhabvala, 1992; Kypta, R.M., unpublished observations) and can be blocked or potentiated by tyrosine kinase inhibitors, depending on the cell type (Bixby and Jhabvala, 1992; Williams et al., 1994; Kypta, R.M., unpublished observations). Thus, a dynamic balance of phosphorylation and dephosphorylation of β-catenin may regulate cadherin-dependent cell interactions.

There are several ways in which phosphorylation of β-catenin might regulate cell function. For example, tyrosine phosphorylation of β-catenin may result in changes in the associations of components in the cadherin–catenin complex. Consistent with this possibility, transformation of epithelial cells by ras, which increases tyrosine phosphorylation of β-catenin, results in dissociation of β-catenin and cadherin, concomitant with stronger association of p120 Cas with cadherin (Kinch et al., 1995). Tyrosine phosphorylation of β-catenin may also regulate its ability to signal; in Drosophila, Wingless negatively regulates Armadillo phosphorylation on serine and tyrosine residues (Peifer et al., 1994). An understanding of the pathways that lead to dephosphorylation of β-catenin may help us
distinguish the possible functions of β-catenin tyrosine phosphorylation.

In this paper, we show that β-catenin is subject to rapid dephosphorylation, both in vitro and in vivo. We have investigated the mechanism for this dephosphorylation and found that the cadherin–catenin complex is associated with a member of the leukocyte common antigen-related protein (LAR)1 subfamily of transmembrane tyrosine phosphatases (LAR-PTP). There are three known LAR-PTPs (LAR [Streuli et al., 1988], PTP8 [Krueger et al., 1990; Mizuno et al., 1993], and PTP-P1 [Pan et al., 1993]), also known as PTP NE-3, RPTPα, CRYPα, and LAR-2 [Walton et al., 1993; Yan et al., 1993; Stoker, 1994; Zhang et al., 1994]), all of which have extracellular domains consisting of immunoglobulin-like domains and fibronectin type III domains, and at least two of which are expressed in PC12 cells. Our data suggest potential mechanisms by which cadherin-dependent processes might be regulated by tyrosine dephosphorylation.

Materials and Methods

Cell Culture, Labeling, and Transfection

The PC12 cell line expressing human trk, PC12 6-24 (from Dr. D. Kaplan, McGill University, Montreal, Quebec) was grown according to Hempstead et al. (1992). Cells were incubated for 16 h in low-serum medium (LSM: 10% normal growth medium in DME) in the presence or absence of 20 ng/ml NGF (from Dr. W. Moberly) before lysis and immune precipitation. For Fig. 2 a, cells were incubated for 4 h in methionine and cysteine-free growth medium containing dicylized serum and 1 mM/ml Translabel (ICN Biomedical, Inc., Costa Mesa, CA). PC12 cells were grown on collagen (Vitrogen, Celtrix Labs, Palo Alto, CA), in DME containing 10% horse serum and 5% FCS. PC9 cells (kindly provided by M. Takeichi, Kyoto University, Japan) were grown in a 1:1 mixture of DME and Ham’s F12 supplemented with 10% FCS.

The COS cells were grown in DME containing 10% FCS. Transient transfections were done in OptiMem 1 using Lipofectamine (GIBCO BRL, Gaithersburg, MD) according to manufacturer’s protocols. Cells were transfected in serum-free medium for 6 h and then grown for 16 h in OptiMem 1 with 10% FCS, followed by 8–10 h in DME with 10% FCS and finally 18–24 h in DME with 1% FCS.

Antibodies

Antibodies recognizing β-catenin were generated as follows: Xenopus β-catenin cDNA (McCrea et al., 1991) (from Drs. P. McCrea, MD Anderson Cancer Center, Houston, TX, and B. Gumbiner, Sloan-Kettering Cancer Center, New York) was expressed in Sf9 cells using a baculovirus expression system (Clontech, Palo Alto, CA) and purified on NTA-agarose (Qiagen, Inc., Chatsworth, CA) by means of six carboxyl-terminal histidine residues generated by PCR. (PCR-generated fragments were either sequenced or replaced by sequences from the original β-catenin clone.) Purified protein was injected into rabbits for production of antiserum (CALTAG, San Francisco, CA). Anti-α-catenin antibodies were generated in rabbits using as immunogen the peptide CQOKHISPQVALEFKAMDSF, corresponding to the carboxyl-terminal residues of chicken α-catenin, coupled to maleimide-activated keyhole limpet hemocyanin (KLH; Pierce, Rockford, IL). Anti-β-catenin antibodies were raised in rabbits using as immunogen the peptide CYQACLEYLGSSFDHYAT coupled to KLH. The control antibody used was raised in rabbits against the control peptide (CDQYKPCYEVALYLENNSG) coupled to KLH. Monoclonal antibodies to human LAR and receptor protein tyrosine phosphatase RPTPα were kindly provided by Dr. M. Streuli (Dana-Farber Cancer Institute, Boston, MA). Antibodies used for Western blotting are commercially available (see below).

DNA Plasmid Constructs

The β-catenin constructs were generated as follows: The coding region of Xenopus β-catenin cDNA (McCrea et al., 1991) was amplified by PCR to include a sequence encoding a myc-epitope tag (EQKLIPLSEDLD) at the carboxyl-terminus. The PCR-generated construct was ligated into pcRBI and the ends were sequenced. Amplified DNA that was not sequenced was replaced by subcloning of unamplified fragments from the original β-catenin clone. An NsiI fragment encoding the myc-tagged β-catenin (βmyc) was subcloned into the PstI site of the COS cell expression vector pMT23 (from Dr. Gordon Wong, Genetics Institute, Cambridge, MA). βCMyc was generated by deleting the sequences upstream of the unique XhoI site, thereby encoding a protein beginning at methionine 174 of βmyc. βCMyc comprises residues 1–698 of β-catenin with a myc tag. It was generated by deletion mutagenesis using the Transformant Mutagenesis Kit (Clontech, Palo Alto, CA) and a mutagenic oligonucleotide that removed the sequences encoding residues 699–781. βCMyc encodes residues 173–698 of β-catenin with a myc tag and was generated by deleting the sequences upstream of the unique XhoI site in βCMyc. βCMyc was generated by deleting the sequences between the unique XhoI and XbaI sites in βCMyc. It encodes a protein containing residues 1–151 fused to residues 648–781 followed by the myc-epitope tag. Rat TrkA cDNA (kindly provided by Dr. D. Clary, SUGEN, Redwood City, CA) was also subcloned into pMT23 in sense and antisense orientations. Human LAR (Streuli et al., 1988) and RPTPα (Palido et al., 1995a) cDNA expression vectors were kindly provided by Dr. M. Streuli.

Cell Lysis and Immune Precipitation

All reagents were from Sigma Chemical Co. (St. Louis, MO) unless specified. Sodium orthovanadate was prepared as described (Brown and Gordon, 1984) and added to 0.1 mM at 37°C. Before lysis, cells (~2 × 10⁶ per 10-cm plate) were washed once in PBS, except for experiments involving phosphatase assays, where they were washed in Tris-buffered saline (TBS: 50 mM Tris-Cl, pH 7.5, 150 mM NaCl) with 0.1 mM DTT (10% normal growth medium in DME) in the presence or absence of 20 ng/ml NGF (from Dr. W. Moberly) before lysis and immune precipitation. For Fig. 2 a, cells were incubated for 4 h in methionine and cysteine-free growth medium containing dicylized serum and 1 mM/ml Translabel (ICN Biomedical, Inc., Costa Mesa, CA). PC12 cells were grown on collagen (Vitrogen, Celtrix Labs, Palo Alto, CA), in DME containing 10% horse serum and 5% FCS. PC9 cells (kindly provided by M. Takeichi, Kyoto University, Japan) were grown in a 1:1 mixture of DME and Ham’s F12 supplemented with 10% FCS.

Tissues were prepared at 4°C by incubating with 8 μl antiserum for 1 h, followed by incubation for 30 min at 4°C on a rotator with protein A-Sepharose. Immunoprecipitates were washed four times in lysis buffer and once in TBS containing 0.1 mM vanadate.

Phosphatase Assays

Cells were washed and lysed using LB with molybdate and vanadate, but with 10 mM DTT. Immune complexes were washed four times in LB and once in phosphatase buffer (20 mM Tris-Cl, pH 6.8, 10 mM DTT). Phosphatase assays were conducted in 50 μl phosphatase buffer with or without 0.1 mM vanadate and 0.1 mM phosphopeptide DADEPYLIPOQQ (Promega Corp., Madison, WI). The serine-phosphorylated peptide used in some control experiments was GENPIYPKSAVTTVNVKYEYGK, a
sequence which corresponds to the cytoplasmic tail of integrin β1 subunit (a gift from D.Y. Wu, University of California, San Francisco, CA). For peptide dephosphorylation, immune complexes were prepared using 200 μg extract, and phosphate released was measured using malachite green according to manufacturers instructions (Promega Corp.). Reactions were approximately linear over the time assayed. Control immune precipitates were done using preimmune serum, rabbit IgG, or antisera raised to unrelated peptides, and all gave similar results.

**Western Blotting**

Washed immune complexes were heated to 100°C for 5 min in reducing SDS sample buffer, separated on 6% polyacrylamide gels, and transferred to nitrocellulose (BA83; Schleicher & Schuell, Inc., Keene, NH) using a semi-dry apparatus (Millipore Corp., Bedford, MA). Membranes were rinsed in 10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.1% Tween-20 (TBS-T) and incubated in blocking buffer (TBS-T with 5% BSA, 1% ovalbumin) for 1 h. Primary antibodies were diluted in blocking buffer and incubated with membranes for 2-16 h. Anti-β-actin antibodies were used at 1:100, a rat monoclonal antibody to n-catenin (α-18 from Drs. A. Nagafuchi and S. Tsukita) (Nagafuchi and Tsukita, 1994) at 1:20, β-catenin monoclonal antibody (Transduction Laboratories, Lexington, KY) at 1:1,000, pan-cadherin polyclonal and monoclonal antibodies (Sigma Chemical Co.) at 1:500, anti-myc epitope tag monoclonal antibody (9E10; Santa Cruz Biotech. Inc., Santa Cruz, CA) at 1:1,000, and HRP-conjugated antiphosphotyrosine antibody RC20H (Transduction Laboratories) at 1:2,000. Membranes were washed in TBS-T and probed with either HRP-conjugated (GIBCO BRL) or alkaline phosphatase-conjugated (Promega Corp.) secondary antibodies according to manufacturers instructions. Blots were developed using chemiluminescence for HRP-conjugated antibodies (ECL; Amersham Corp., Arlington Heights, IL), or using 160 μg/ml 4-nitro blue tetrazolium chloride (NBT) and 80 μg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100 mM NaCl, 5 mM MgCl₂, 100 mM Tris, pH 9.5, for alkaline phosphatase-conjugated antibodies. Where indicated in the figure legends, blots were stripped by incubation at 65°C in 62.5 mM Tris-Cl, pH 6.8, 0.1 M β-mercaptoethanol, 2% SDS for 10 min, followed by washing overnight with several changes of TBS-T before reprobing.

**Results**

**β-Catenin Immune Precipitates Contain Tyrosine Phosphatase Activity**

In experiments directed towards understanding the regulation of N-cadherin function in neurons, we have been studying tyrosine phosphorylation of β-catenin in PC12 6–24 cells. Since these cells overexpress human TrkA, NGF treatment results in enhanced and prolonged phosphorylation of intracellular proteins (Hempstead et al., 1992). To our surprise, we observed a similar level of β-catenin tyrosine phosphorylation in PC12 6–24 cells and normal PC12 cells (Kypta, R., unpublished observations), suggesting that a tyrosine phosphatase (PTP) acting preferentially on β-catenin may compensate for the increase in TrkA activity.

**Figure 1.** Phosphotyrosine phosphatase activity associated with β-catenin. (a) Anti-phosphotyrosine (upper panel) or anti-β-catenin (lower panel) Western blots of β-catenin immune precipitates from equal amounts of extracts of PC12 6–24 cells either untreated (lane 1), treated with 50 ng/ml NGF for 5 min (lane 2), treated with 0.1 mM sodium orthovanadate for 30 min (lane 3), treated with 50 ng/ml NGF for 16 h (lane 4), or treated with NGF for 16 h and sodium orthovanadate for the final 30 min (lane 5). For the result shown in the lower panel, the blot in the upper panel was stripped and reprobed with anti-β-catenin antibodies. The lines on the left of each panel indicate the positions of molecular mass markers (100.6 kD and 71.6 kD). (b) Anti-phosphotyrosine (upper panel) or anti-β-catenin (lower panel) Western blots β-catenin immune precipitates from equal amounts of extracts of cells treated with NGF for 16 h and sodium orthovanadate for the final 30 min and lysed in the absence of vanadate. Washed immune precipitates were incubated in phosphatase buffer with vanadate (lane 1) or without vanadate (lane 2). For the result shown in the lower panel, the blot in the upper panel was stripped and reprobed with anti-β-catenin antibodies. The residual signal in lane 2 is similar to that seen for purified unphosphorylated β-catenin. (c) Phosphatase activity in control antibody or β-catenin immune precipitates as measured using a phosphotyrosyl peptide substrate. In this experiment, 60 pmol of phosphate corresponded to 100 U (an OD of 0.1 at 620 nm after addition of malachite green reagent).
To investigate the possible role of a tyrosine phosphatase, we examined the effects on β-catenin phosphorylation of the tyrosine phosphatase inhibitor, sodium orthovanadate (Brown and Gordon, 1984). In the absence of vanadate, addition of NGF to PC12 6–24 cells for 10 min (Fig. 1 a, lane 2) or 16 h (lane 3) increased tyrosine phosphorylation of β-catenin. Interestingly, addition of vanadate to PC12 6–24 cells also increased tyrosine phosphorylation of β-catenin, even in the absence of NGF (lane 4). β-Catenin phosphorylation was dependent on TrkA activity since the effects of vanadate were blocked by the TrkA inhibitor Tyrphostin AG879 and by K252a (data not shown). The latter is a relatively broad specificity kinase inhibitor that nonetheless inhibits purified TrkA in vitro and blocks the effects of NGF (but not FGF) on PC12 cells in vivo (Berg et al., 1992; Nye et al., 1992; Tapley et al., 1992; Ohmichi et al., 1993). Addition of vanadate to PC12 6–24 cells that had been treated with NGF for 16 h resulted in dramatically elevated phosphorylation of β-catenin (lane 5). Increased tyrosine phosphorylation of β-catenin after addition of vanadate also was observed using α-catenin immune precipitates and was detected in another cell line which expresses human TrkA (PC12 6–15 cells; Hempstead et al., 1992) (data not shown). The changes in β-catenin tyrosine phosphorylation were not due to changes in β-catenin levels, as shown by reprobing the blot with anti–β-catenin antibodies (Fig. 1 a, lower panel).

The increased phosphorylation of β-catenin in cells treated with NGF or vanadate and the inhibition of this phosphorylation by inhibitors of TrkA suggest that β-catenin phosphorylation is tightly regulated by the activities of TrkA and a tyrosine phosphatase. To test the possibility that β-catenin is associated with a tyrosine phosphatase, immune precipitates containing tyrosine-phosphorylated β-catenin were prepared from extracts of PC12 6–24 cells that had been treated with NGF and vanadate (which was removed before lysis). Washed immune precipitates were incubated at 30°C either with or without vanadate and then analyzed by anti-phosphotyrosine Western blotting. The results in Fig. 1 b show that the amount of phosphoryl-  

The Cadherin–Catenin Complex Associates with LAR-related Phosphatases

We sought to identify the tyrosine phosphatase associated with the cadherin–catenin complex. There are several candidates that could play this role: the SH2-containing protein tyrosine phosphatases SHP-1 and SHP-2, both of which are expressed in PC12 cells (Vambutas et al., 1995), are cytoplasmic phosphatases that bind tyrosine-phosphorylated proteins. SHP-1 is activated by NGF treatment (Vambutas et al., 1995); RPTPμ is a transmembrane tyrosine phosphatase that shares weak sequence homology with cadherins (Tonks et al., 1992) and appears to associate directly with cadherins (Brady-Kalnay et al., 1995); finally, at least two members of the LAR subfamily of trans- membrene tyrosine phosphatases are expressed in PC12 cells, and their mRNA levels are increased in NGF-treated PC12 cells (Pan et al., 1993; Zhang and Longo, 1995).

We were unable to detect association between SHP-1 or SHP-2 with the cadherin–catenin complex, and RPTPμ is not expressed in PC12 cells (Kypta, R.M., unpublished observations; Gebbink et al., 1995). To examine the possibility that LAR-PTPs mediate dephosphorylation of β-catenin, we prepared an antibody (called δ-end) to a peptide corresponding to the common carboxyl-terminal tail of the LAR subfamily of PTPs. δ-end immune precipitates from radiolabeled PC12 6–24 cell extracts contained at least four specific proteins, with apparent molecular masses of approximately 150, 105, 95, and 80 kD (Fig. 2 a, lane 2). These proteins were not present when the antiserum was incubated with cell extracts in the presence of the δ-end peptide (lane 1). In other experiments, the 150- and 80-kD proteins could further be resolved into multiple bands, and the 80-kD proteins were recognized in Western blots using the δ-end antiserum (data not shown). These results are consistent with previous studies using antibodies to LAR and PTPβ, which have shown that the mature forms of these phosphatases consist of a 150-kD extracellular fragment (E subunit) and a noncovalently associated 85-kD fragment (P subunit) containing extracellular, transmembrane, and cytoplasmic sequences (Streuli et al., 1992; Yu et al., 1992; Pulido et al., 1995a). For convenience, the LAR subfamily proteins recognized by the antibody δ-end will be called LAR-PTPs in this paper. Interestingly, the two additional major radiolabeled proteins specifically immune precipitated by δ-end antibodies (Fig. 2 a, lane 2) comigrated with β- and α-catenins (Fig. 2 a, lanes 4 and 6, respectively), suggesting that they might be LAR-PTP-associated catenins.

To provide stronger evidence for association of LAR-PTPs and catenins, δ-end immune precipitates were probed with antibodies to components of the cadherin–catenin complex. The results demonstrated the presence of α-catenin (Fig. 2 b, lane 2), cadherin (Fig. 3), and β-catenin (Fig. 4). Results of an anti-α-catenin Western blot (Fig. 2 b) show that α-catenin was detected in δ-end immune precipitates prepared in the presence of an irrelevant control peptide (lane 2) but not in δ-end immune precipitates prepared in the presence of the immunogen, the δ-end peptide (lane 1). In addition, control immune precipitates (lanes 3 and 4) did not contain α-catenin, and the presence of α-catenin in anti–α-catenin immune precipitates was not affected by incubation with either δ-end (lane 5) or control (lane 6) peptides.

Comparison of the relative amounts of cadherin detected in α-catenin and δ-end immune precipitates (Fig. 3 a) suggested that the amount of cadherin associated with
Figure 2. Association of the cadherin–catenin complex with LAR-related PTPase proteins. (a) Immune precipitates from equal amounts of [35S]methionine/cysteine-labeled PC12 6–24 cell extracts were visualized by autoradiography: Anti-δ-end in the presence of δ-end peptide (lane 1), anti-δ-end (lane 2), anti-β-catenin in the presence of purified β-catenin (lane 3), anti-β-catenin (lane 4), anti-α-catenin in the presence of cognate peptide (lane 5), and anti-α-catenin (lane 6). The lines without arrows on the right indicate the positions of molecular mass markers (100.6 kD and 71.6 kD). The positions of proteins specifically immune-precipitated by anti-δ-end antibodies are indicated by the arrows on the left. The fastest migrating band corresponds to LAR-PTP P subunits. The positions of α-catenin (lane 6), β-catenin (lane 4), and the LAR-PTP P subunit (lane 2) were confirmed by transfer of radiolabeled immune precipitates to nitrocellulose and subsequent Western blotting. In lane 3, immune precipitation using anti-β-catenin antibodies in the presence of purified unlabeled β-catenin blocked association of the antibody with labeled β-catenin but not with α-catenin, probably because α-catenin bound to the purified β-catenin in the immune precipitate. (b) The following immune precipitates from equal amounts of cell extracts were probed with anti-α-catenin antibody: Anti-δ-end in the presence of either δ-end peptide (lane 1) or control peptide (lane 2), anti-control peptide in the presence of either δ-end peptide (lane 3) or control peptide (lane 4), and anti-α-catenin in the presence of either δ-end peptide (lane 5) or control peptide (lane 6). The lines on the left indicate the positions of molecular mass markers (208 kD, 100.6 kD, and 71.6 kD).

Figure 3. Association of LAR-PTPs with cadherin. (a) The following immune precipitates from equal amounts of cell extract were probed with anti-pan cadherin antibody: Control anti-peptide antibody (lane 1), anti-δ-end (lane 2), anti-α-catenin (lanes 3 and 4; lane 3 contained 20 times more immune precipitate than lane 4). Lane 5 contains cell extract. The lines on the left indicate the positions of molecular mass markers (208 kD, 100.6 kD, and 71.6 kD). (b) Cell extracts depleted using the following antibodies were loaded in equal amounts (except where indicated) and probed using anti-pan cadherin antibodies: Control anti-peptide antibody (lane 1), anti-δ-end (lanes 4 and 5), and anti-α-catenin (lanes 6 and 7). Lanes 2, 3, and 8 contained 90%, 80%, and 20%, respectively, of the amount of extract loaded in lane 1. The lines on the left indicate the positions of molecular mass markers (208 kD, 100.6 kD, and 71.6 kD).

α-catenin (lane 3) was about 10 times that associated with LAR-PTPs (lane 2). For comparison, lane 4 contains 20 times less α-catenin immune precipitate than lane 2. To determine the fraction of total cadherin associated with LAR-PTPs, we depleted PC12 cell extracts using either control, α-catenin, or δ-end antibodies, and measured the amount of cadherin remaining (Fig. 3 b). The results from duplicate experiments were quantitated by densitometry and comparison with known amounts of cell extracts that had been incubated with control antibodies. While α-catenin antibodies depleted over 80% of cadherin (lanes 6 and 7), δ-end antibodies depleted 8%. Therefore, about 10% of cadherin–catenin complexes in PC12 cells are associated with LAR-PTPs.

Octylglucoside, but not NP-40, has been shown to selectively dissociate α-catenin from the cadherin–catenin complex (Ozawa and Kemler, 1992). We compared the association of LAR-PTPs with catenins after lysis in buffers containing either NP-40 or octylglucoside (Fig. 4). Cell extracts prepared in NP-40 lysis buffer (lane 7) and octylglucoside lysis buffer (lane 8) contained similar amounts of α-catenin (Fig. 4, upper panel, lanes 7 and 8), β-catenin (lower panel, lanes 7 and 8), and LAR-PTPs (data not shown). As anticipated, preparation of PC12 cell extracts in octylglucoside, but not NP-40, disrupted association of α-catenin with cadherin (upper panel, lanes 3 and 4) and β-catenin (lanes 5 and 6). Interestingly, the association of LAR-PTPs with α-catenin was also disrupted after lysis in octylglucoside but not NP-40 (upper panel, lanes 1 and 2). Reprobing the membranes with anti-β-catenin antibodies (Fig. 4, lower panel) indicated that, while octylglucoside did not affect the association between β-catenin and cadherins (lanes 3 and 4), it disrupted the association between β-catenin and LAR-PTPs (lanes 1 and 2). Since octylglucoside disrupts the association of LAR-PTPs with both α-catenin and β-catenin, this experiment does not demon-
strate which protein in the cadherin-catenin complex mediates the interaction of this complex with LAR-PTPs.

The Amino-terminal Domain of β-Catenin Is Required for Association of the Cadherin-Catenin Complex with LAR-PTPs

To determine which component of the cadherin-catenin complex mediates the interaction with LAR-PTPs, we constructed a number of myc-epitope-tagged β-catenin deletion mutants and expressed them transiently in COS cells (Fig. 5). The upper panel of Fig. 5 is an anti-myc (9E10) Western blot of whole cell extracts containing these constructs. We previously observed that LAR-PTPs associate with the cadherin-catenin complex in some fibroblast cell lines, including COS cells, although to a lesser extent than in PC12 cells (Kypta, R.M., data not shown). Therefore, β-end immune precipitates were prepared from COS cell extracts that contained β-catenin deletion mutants and then probed with antibodies that recognize the myc-epitope tag (Fig. 5, lower panel). As expected, full-length, myc-epitope-tagged β-catenin (β) associated with both LAR-PTPs (lower panel, lane 1) and α-catenin (data not shown). Deletion of the amino-terminal domain (ΔN, lane 2) but not the carboxyl-terminal domain (ΔC, lane 3) of β-catenin prevented association of β-catenin with both LAR-PTPs (lower panel) and with α-catenin (data not shown). Interestingly, a β-catenin mutant lacking the armadillo repeat region (ΔR) still associated with LAR-PTPs (lower panel, lane 5).

Since cadherins associate with the armadillo repeat region of β-catenin, these results indicate that, in contrast to RPTPs, LAR-PTPs do not interact directly with cadherins. However, these experiments do not address whether the amino-terminal domain of β-catenin directly binds LAR-PTPs or if the association is mediated by α-catenin (or an unknown protein). To determine the importance of α-catenin to the association between LAR-PTPs and the cadherin-catenin complex, we prepared extracts from PC9 cells (Fig. 6). PC9 cells do not express α-catenin, but do express β-catenin and cadherins (Hirano et al., 1992). As expected, anti-α-catenin antibodies communiprecipitated β-catenin from extracts of PC12 cells (lane 2), but not from extracts of PC9 cells (lane 5). Interestingly, β-catenin was found in β-end immune precipitates from both PC12 cells (lane 3) and PC9 cells (lane 6).

To summarize, these results indicate that LAR-PTPs associate with the cadherin-catenin complex through an interaction with the amino-terminal domain of β-catenin and that β-catenin does not have to associate with cadherins or α-catenin to mediate this interaction.

Figure 5. The amino-terminal domain of β-catenin is required for association of LAR-PTPs with the cadherin-catenin complex. Anti-myc tag (9E10) Western blots of extracts (upper panel) and anti-β-end immune precipitates (lower panel) from extracts of COS cells transfected with the indicated β-catenin expression constructs. β, full-length myc-tagged β-catenin (lane 1); ΔN, residues 174-781 (lane 2); ΔC, residues 1-698 (lane 3); ΔNΔC, residues 174-698 (lane 4); ΔR, residues 1-151 fused to residues 648-781 (lane 5), or cells transfected with empty vector (lane 6). The arrows indicate the positions of β (lane 1), ΔC (lane 3), and ΔR (lane 5). The lines on the left of each panel indicate the positions of molecular mass markers (100.6 kD and 71.6 kD). The bands running above the position of the bottom arrow are IgG heavy chain, which cross-reacts with the secondary antibody.
Figure 6. The association between LAR-PTPs and the cadherin-catenin complex does not require α-catenin. Anti-β-catenin Western blot of immune precipitates from PC12 cells (lanes 1-3) or PC9 cells (lanes 4-6). Antibodies used for immune precipitation were anti-β-catenin (lanes 1 and 4), anti-α-catenin (lanes 2 and 5), and anti-δ-end (lanes 3 and 6). The lines on the left of each panel indicate the positions of molecular mass markers (100.6 kD and 71.6 kD).

TrkA-dependent Tyrosine Phosphorylation of LAR-PTPs

Tyrosine phosphorylation of β-catenin might be expected to alter its interaction either with LAR-PTPs, cadherin, or other catenins. However, we have not observed such changes after vanadate treatment of PC12 6–24 cells (data not shown). As another possibility, activation of TrkA may result in changes in LAR-PTP catalytic activity, perhaps by tyrosine phosphorylation of the latter. We therefore examined the possibility that LAR-PTPs are tyrosine phosphorylated. Results in Fig. 7 show that addition of vanadate to NGF-treated PC12 6–24 cells increased tyrosine phosphorylation of the P subunit of LAR-PTPs (lane 4) and β-catenin (lane 2). This phosphorylation was blocked by the TrkA inhibitors K252a and tyrphostin AG879 (data not shown), suggesting that TrkA activation is necessary for tyrosine phosphorylation of LAR-PTPs. We were unable to detect changes in LAR-PTP tyrosine phosphorylation or catalytic activity after addition of NGF alone (Kypta, R.M., unpublished observations), perhaps because LAR-PTPs are rapidly dephosphorylated in the absence of tyrosine phosphatase inhibitors. It thus remains possible that LAR-PTP activity is regulated by tyrosine phosphorylation, but that this was not detectable using in vitro assays.

To determine whether TrkA induces tyrosine phosphorylation of LAR-PTPs without the addition of vanadate to living cells, LAR-PTPs were coexpressed with TrkA in COS cells (Fig. 7 b). COS cells were transfected with cDNAs expressing human LAR (lanes 1 and 2) and human RPTP6 (lanes 3 and 4) together with plasmids containing rat TrkA cDNA (lanes 2 and 4) or TrkA cDNA cloned in the antisense orientation as a control (lanes 1 and 3). LAR and RPTP6 immune precipitates from transfected COS cell extracts were probed sequentially with antibodies to anti-phosphotyrosine (upper panel) and δ-end antibodies (lower panel). Expression of TrkA induced tyrosine phosphorylation of both LAR (upper panel, lane 2) and RPTP6 (lane 4). Therefore, LAR-PTPs can be tyrosine-phosphorylated without the addition of vanadate.

Figure 7. TrkA induces tyrosine phosphorylation of LAR-PTPs. (a) Tyrosine phosphorylation of LAR-PTPs in PC12 6–24 cells. Western blots of immune precipitates from equal amounts of cell extracts of NGF-treated cells that were either untreated (lanes 1 and 3) or treated with vanadate (lanes 2 and 4) before lysis, prepared using anti-β-catenin (lanes 1 and 2) or anti-δ-end (lanes 3 and 4) antibodies, were probed with anti-phosphotyrosine antibodies (upper panel) or anti-δ-end antibodies (lower panel). The positions of LAR-PTP subunits and β-catenin are indicated. The lines on the left of each panel indicate the positions of molecular mass markers (upper panel: 100.6 kD and 71.6 kD; lower panel: 208 kD, 100.6 kD, and 71.6 kD). Tyrosine-phosphorylated β-catenin is detectable in δ-end immune precipitates (upper panel, lane 4). Vanadate does not alter the amount of LAR-PTPs (lower panel, compare lanes 3 and 4). (b) TrkA expression in COS cells induces tyrosine phosphorylation of LAR and RPTP6. Anti-phosphotyrosine (upper panel) and anti-δ-end (lower panel) Western blots of anti-LAR (lanes 1 and 2) or anti-RPTP6 (lanes 3 and 4) immune precipitates from equal amounts of cell extracts of COS cells transfected with vectors expressing LAR cDNA (lanes 1 and 2), RPTP6 cDNA (lanes 3 and 4), control DNA (lanes 1 and 3), or TrkA cDNA (lanes 2 and 4). In each panel, the arrows mark the positions of uncleaved LAR-PTP P+E subunits (top arrows) and cleaved LAR-PTP P subunits (bottom arrows). The lines on the right of each panel indicate the positions of molecular mass markers (208 kD, 100.6 kD, and 71.6 kD). The additional band recognized by anti-δ-end antibodies (lower panel, lanes 3 and 4) may be a breakdown product of RPTP6, since it was observed only in COS cells transfected with RPTP6 cDNA.
Regulation of the LAR-PTP-cadherin-catenin complex by NGF. (a) NGF increases cellular levels of LAR-PTPs. Western blots of extracts (lanes 1–6) or δ-end immune precipitates (lanes 7 and 8) from untreated PC12 6–24 cells (lanes 1, 3, 5, and 7) or PC12 6–24 cells treated with NGF for 16 h (lanes 2, 4, 6, and 8) were probed with anti-pan cadherin (lanes 1 and 2), anti-α-catenin (lanes 3 and 4), anti-β-catenin (lanes 5 and 6), and anti-δ-end (lanes 7 and 8) antibodies. The lines on the right of lanes 1–5 and on the left of lanes 7 and 8 indicate the positions of molecular mass markers (208 kD, 100.6 kD, and 71.6 kD). These blots were developed using a colorimetric assay. (b) NGF treatment results in downregulation of the LAR-PTP-cadherin-catenin complex. Western blots of immune precipitates from equal amounts of cell extracts of untreated cells (lanes 1, 3, 5, and 8) or cells treated with NGF for 16 h (lanes 2, 4, 6, and 7) using anti-α-catenin (lanes 1 and 2), anti-β-catenin (lanes 3 and 4), and anti-δ-end (lanes 5–8) antibodies were probed with anti-β-catenin monoclonal antibody (lanes 1–5) or both anti-β-catenin and anti-δ-end antibodies (lanes 7 and 8, developed using a colorimetric assay). The lines on the left of the panel indicate the positions of molecular mass markers (100.6 kD and 71.6 kD).
The rapid increase of β-catenin tyrosine phosphorylation in vanadate-treated PC12 6–24 cells strongly suggests that this association also occurs in vivo.

The association of the major components of the cadherin–catenin complex was not detectably altered after vanadate-induced tyrosine phosphorylation of β-catenin (data not shown), as was observed by others using v-src–transformed cells (Behrens et al., 1993). However, transformation of epithelial cells by ras, which increases tyrosine phosphorylation of β-catenin, results in dissociation of β-catenin and cadherin (Kinch et al., 1995). Therefore, there may be cell-type specific differences in the regulation of cadherin–catenin interactions by tyrosine phosphorylation. These might be determined by phosphorylation of specific tyrosine residues on β-catenin or by phosphorylation of other proteins.

One possible result of increased tyrosine phosphorylation of β-catenin is a reduction in cadherin-dependent cell aggregation. We have been unable to test this possibility in PC12 cells, since they aggregate poorly (Kypka, R.M., unpublished observations). Recently, expression of v-src in fibroblasts lacking β-catenin was shown to shift cell adhesion mediated by a cadherin–α-catenin fusion protein from a strong to a weak state, suggesting that tyrosine phosphorylation of β-catenin is not required for this shift (Takeda et al., 1995). Since cadherin-dependent cell aggregation in these fibroblasts is the same in the presence or absence of v-src, tyrosine phosphorylation of β-catenin may regulate cadherin function of cells already in the weak state of cell adhesion, such as PC12 cells, during processes such as neurite outgrowth.

Recently Brady-Kalnay et al. (1995) reported that cadherins associate with the transmembrane tyrosine phosphatase RPTPμ in a mink lung epithelial cell line. While our experiments do not address the possibility that the cadherin–catenin complex associates with tyrosine phosphatases in addition to LAR-PTPs in PC12 cells, the activity measured in β-catenin immune precipitates cannot include RPTPμ, since this protein is not expressed in PC12 cells (Kypka, R.M., unpublished observations; Gebbink et al., 1995). The RPTPμ-related protein, RPTPs (Jiang et al., 1993), is expressed at low levels in PC12 cells (Kypka, R.M., unpublished observations). Therefore, it remains possible that the cadherin–catenin complex in PC12 cells also associates with RPTPks.

There are a number of further differences between the cadherin–catenin complexes that associate with LAR-PTPs and RPTPμ. RPTPμ localizes to cell junctions. In contrast LAR-PTPs are not concentrated in cell junctions of PC12 cells (data not shown) or MCF-7 cells (Serra-Pages et al., 1995); and, while RPTPμ associates directly with cadherins, the association between LAR-PTPs and the cadherin–catenin complex is mediated by the amino-terminal domain of β-catenin.

The importance of the amino-terminal domain of β-catenin for the association of LAR-PTPs with the cadherin–catenin complex is intriguing. While several proteins interact with the armadillo repeat region of β-catenin (Kirkpatrick and Peifer, 1995), only α-catenin has been shown to associate with the amino-terminal domain of β-catenin, and the binding site for α-catenin overlaps with the beginning of the armadillo repeat region (Aberle et al., 1996). Oncogenic forms of β-catenin deleted in the amino-terminal domain have been found (Oyama et al., 1994; Kawanishi et al., 1995; Whitehead et al., 1995). The inability of one of these mutants to bind α-catenin results in reduced cell adhesion, which can be rescued by expression of full-length β-catenin (Kawanishi et al., 1995). It has been suggested that such mutations in β-catenin may be involved in the enhancement of tumor invasion in human cancers (Oyama et al., 1994). Since these mutant forms of β-catenin probably no longer associate with LAR-PTPs, it is also possible that disruption of the cadherin–catenin–LAR-PTP complex plays a role in cell transformation. Consistent with this possibility, human LAR (Streuli et al., 1992) and murine RPTPδ (Mizuno et al., 1993) map to chromosomal regions that contain candidate tumor suppressor genes.

The high sequence homology among LAR-PTPs suggests that all three members associate with the cadherin–catenin complex. Indeed, LAR-PTPs are known to interact with a common cytoplasmic protein, LIP-1 (Pulido et al., 1995b). To date, we have been unable to demonstrate a direct association between the cytoplasmic domain of the rat LAR-PTP PTP-P1 (Pan et al., 1993) and β-catenin using gel overlay assays (Kypka, R.M., unpublished observations), so the interaction may be mediated by another protein. Alternatively, posttranslational modifications of β-catenin or a particular isoform of PTP-P1 may be required for association.

The effects of vanadate on tyrosine phosphorylation of LAR-PTPs in PC12 6–24 cells suggest that LAR-PTPs are rapidly phosphorylated and dephosphorylated in vivo. Expression of TrkA induced tyrosine phosphorylation of LAR and RPTPδ in COS cells without the addition of vanadate before cell lysis. Interestingly, tyrosine phosphorylation of LAR and RPTPδ in COS cells was on their full-length, uncleaved forms, rather than the cleaved P subunits of LAR-PTPs that were phosphorylated in vanadate-treated PC12 6–24 cells. This may reflect the ratios of uncleaved to cleaved forms of LAR-PTPs in COS and PC12 cells. An intriguing possibility is that cleavage of LAR-PTPs leads to their activation, with PC12 cells containing a higher proportion of cleaved, activated LAR-PTPs, whose phosphorylation is only detectable using vanadate.

Assessment of the effects of tyrosine phosphorylation on LAR-PTP activity is hampered by the fact that stable phosphorylation requires the presence of vanadate, which inhibits LAR-PTPs. An understanding of the consequences of this phosphorylation on LAR-PTP activity will require the identification and mutation of the TrkA-induced tyrosine phosphorylation sites. There is precedent for regulation of PTPs by tyrosine phosphorylation: the nonreceptor phosophatase, PTP1C, is phosphorylated on tyrosine in response to insulin receptor activation, and this correlates with an increase in its activity (Uchida et al., 1994); RPTPs is phosphorylated constitutively on tyrosine (den Hertog et al., 1994; Su et al., 1994), and in vitro experiments suggest that tyrosine phosphorylation reduces the activity of this phosphatase (den Hertog et al., 1994). Therefore, TrkA-induced phosphorylation of LAR-PTPs provides a potential mechanism for regulating their activities.

Addition of NGF to PC12 6–24 cells reduces the association between LAR-PTPs and the cadherin–catenin com-
plex, suggesting that TrkA activity somehow regulates this association. Consistent with this possibility is the observation that tyrosine phosphatase activity in β-catenin immune precipitates from parental PC12 cells, which express far less TrkA than PC12 6–24 cells, is higher than that in PC12 6–24 cells (Kypta, R.M., unpublished observations). It is unclear whether the reduced association seen after NGF treatment reflects a stoichiometric change in binding or a weakened association that leads to disruption of the complex during cell lysis and immune precipitation. An alternative explanation for the reduced association in NGF-treated cells is that the cadherin–catenin complex associates with a modified form of LAR-PTPs, such as one containing a differentially spliced exon. This is an exciting possibility, since isoforms of LAR, RPTPα, and RPTPβ all contain additional cytoplasmic residues encoded by such exons (Pulido et al., 1995b), which, at least in LAR, become downregulated after NGF treatment of PC12 cells (Zhang and Longo, 1995).

Recently DLAR, a Drosophila LAR-PTP expressed in developing neurons, was shown to play a key role during motor neuron growth cone guidance (Krueger et al., 1996). Although all neurons express DLAR, only a subset of neurons make axon-pathfinding errors in Dlar mutant embryos. While this observation may reflect the restricted localization of the putative ligand for DLAR, it may also delineate specific cadherin-dependent guidance pathways. N-cadherin–dependent neurite outgrowth is affected by inhibition of tyrosine phosphorylation (Bixby and Jhavvala, 1992; Williams et al., 1994). In addition, neurite outgrowth of PC12 cells growing on purified N-cadherin requires NGF and is blocked both by TrkA inhibitors and vanadate (Kypta, R.M., unpublished observations), suggesting that it requires tyrosine kinase and phosphatase activities. Our immunocytochemical experiments (Kypta, R.M., unpublished observations) indicate that LAR-PTPs and cadherins/catenins partially colocalize in neurites, further suggesting a role for the complex in neurite outgrowth. Indeed, the chicken LAR-PTP, CRYPα, which is expressed within both the lamellipodia and filopodia of neuronal growth cones, has been proposed to regulate growth cone migration (Stoker et al., 1995).

The LAR-PTP–cadherin-catenin complex is most abundant in PC12 cells but is also present in fibroblasts, suggesting that it may also have a more general function. We propose that cycles of β-catenin tyrosine phosphorylation and dephosphorylation provide a mechanism rapidly to regulate cell–cell interactions, which may be required for cadherin-dependent functions that include neurite outgrowth (Bixby et al., 1988; Matsunaga et al., 1988; Tomasselli et al., 1988; Bixby and Jhavvala, 1990) and cell motility (Hamaguchi et al., 1993; Nagafuchi et al., 1994; Shibamoto et al., 1994). An alternative function for the LAR-PTP–cadherin-catenin complex might be to regulate the signaling function of β-catenin. For example, LAR-PTPs may be involved in transducing Wnt signals.

There are three, not mutually exclusive ways in which such mechanisms might operate. First, catenins could serve as a link between LAR-PTPs and the actin-based cytoskeleton. This would provide a mechanism to stabilize potential adhesive interactions mediated by the extracellular domains of LAR-PTPs. Second, co-clustering of cadherins and LAR-PTPs may result in activation of LAR-PTPs. This would maintain β-catenin in the phosphorylated state, which may be a prerequisite for cadherin-dependent cell adhesion (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993). Third, association of β-catenin with LAR-PTPs could allow a rapid reversal of growth factor–induced tyrosine phosphorylation of β-catenin. Extracellular signals transduced by binding of putative ligands to LAR-PTPs would then provide additional pathways to modulate cadherin function.

We thank D. Clary, B. Gumbiner, D. Kaplan, P. McCrea, W. Mobley, A. Nagafuchi, M. Streuli, M. Takeichi, and S. Tsukita for reagents; C. Turck for peptide synthesis; M. Günthorpe for oligonucleotides; M. Vivanco for help preparing the figures; A. Weiss, M. Vivanco, and R. Segal for critically reading the manuscript; and members of the lab for discussions and comments.

This work was supported by the Human Frontier Science Programme (R.M. Kypta) and the Howard Hughes Medical Institute.

Received for publication 14 May 1996 and in revised form 18 July 1996.

References
Berg, D.W., L.F. Sternberg, L.F. Pa-

Downloaded from jcb.rupress.org on November 19, 2017