Continuous association of cadherin with β-catenin requires the non-receptor tyrosine-kinase Fer

Gang Xu[1], Andrew W. B. Craig[2], Peter Greer[3], Matthew Miller[1], Panos Z. Anastasiadis[4], Jack Lilien[1,*] and Janne Balsamo[1]

[1]Department of Biological Sciences, The University of Iowa, Iowa City, IA 52242, USA
[2]Department of Biochemistry, Room 641 Botterell Hall, Queen's University, Kingston, Ontario, K7L 3N6, Canada
[3]Department of Pathology, Division of Cancer Biology and Genetics, Room A309 Botterell Hall, Queen's University Cancer Research Institute, Kingston, Ontario, K7L 3N6, Canada
[4]Laboratory of Cell Adhesion and Metastasis, Mayo Clinic, Griffin Cancer Research Building, 4500 San Pablo Road, Jacksonville, FL 32224, USA
*Author for correspondence (e-mail: jack-lilien@uiowa.edu)

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Summary

The function of Type 1, classic cadherins depends on their association with the actin cytoskeleton, a connection mediated by α- and β-catenin. The phosphorylation state of β-catenin is crucial for its association with cadherin and thus the association of cadherin with the cytoskeleton. We now show that the phosphorylation of β-catenin is regulated by the combined activities of the tyrosine kinase Fer and the tyrosine phosphatase PTP1B. Fer phosphorylates PTP1B at tyrosine 152, regulating its binding to cadherin and the continuous dephosphorylation of β-catenin at tyrosine 654. Fer interacts with cadherin indirectly, through p120ctn. We have mapped the interaction domains of Fer and p120ctn and peptides corresponding to these sequences release Fer from p120ctn in vitro and in live cells, resulting in loss of cadherin-associated PTP1B, an increase in the pool of tyrosine phosphorylated β-catenin and loss of cadherin adhesion function. The effect of the peptides is lost when a β-catenin mutant with a substitution at tyrosine 654 is introduced into cells. Thus, Fer phosphorylates PTP1B at tyrosine 152 enabling it to bind to the cytoplasmic domain of cadherin, where it maintains β-catenin in a dephosphorylated state. Cultured fibroblasts from mouse embryos targeted with a kinase-inactivating *fer*^{D743R} mutation have lost cadherin-associated PTP1B and β-catenin, as well as localization of cadherin and β-catenin in areas of cell-cell contacts. Expression of wild-type Fer or culture in epidermal growth factor restores the cadherin complex and localization at cell-cell contacts.

Key words: Cadherin, Fer tyrosine kinase, PTP1B tyrosine phosphatase, β-Catenin, Adhesion

Introduction

Type I classic cadherins are a family of calcium-dependent, homophilic, cell-cell adhesion molecules that have been implicated in many developmental processes, from regional segregation of developing tissues to guidance of axonal projections (reviewed in Tepass et al., 2000; Tepass et al., 2002; Yagi and Takeichi, 2000), as well as tumor growth and metastasis (reviewed in Mareel and Leroy, 2003). This group of cadherins is highly conserved among all vertebrates and is composed of a series of five extracellular repeats with a functionally important HAV sequence in the first repeat, a single transmembrane domain, and a very highly conserved cytoplasmic domain (Nollet et al., 2000). The cytoplasmic domain forms an important functional linkage to the actin cytoskeleton through direct interaction with β-catenin, with α-catenin forming a bridge between β-catenin and actin (reviewed in Pokutta and Weis, 2002; Lilien et al., 2002). These two catenins have been referred to as the 'core' proteins of the cadherin complex, because they appear to be absolutely essential for the stability of type I cadherin-mediated adhesion across wide phyletic boundaries (Pai et al., 1996; Korswagen et al., 2000). There are other proteins associated directly and indirectly with type I cadherins, and these are thought to regulate cadherin function through modulating the formation and/or stability of this core complex, or subsequent steps in stabilizing or modulating cadherin adhesions (reviewed in Lilien et al., 2002).

One correlate of cytoskeletal association is the phosphorylation of tyrosine residues on β-catenin (Balsamo et al., 1996; Balsamo et al., 1998) (reviewed in Lilien et al., 2002). Tyrosine 654 appears to be crucial; phosphorylation of this residue reduces the affinity of β-catenin for cadherin (Roura et al., 1999; Piedra et al., 2001), potentially disrupting their association. Regulation of phosphotyrosine content on β-catenin is a result of the balance between phosphatase and kinase activities immediately available at the cytoplasmic domain of cadherin. Several transmembrane tyrosine phosphatases and the non-receptor tyrosine phosphatase PTP1B are capable of dephosphorylating β-catenin and therefore stabilizing cadherin-mediated adhesions (reviewed in Lilien et al., 2002). However, PTP1B is the only phosphatase that has been shown to regulate cadherin-mediated adhesion by binding directly to the cytoplasmic domain of N-cadherin and dephosphorylating β-catenin (Balsamo et al., 1996; Balsamo et al., 1998; Rhee et al., 2002; Xu et al., 2002). The binding of PTP1B to the cytoplasmic domain of N-cadherin requires the phosphorylation of PTP1B on tyrosine 152 (Rhee et al., 2001). It is interesting that the

binding site for PTP1B is adjacent to and partially overlaps with the site for β-catenin (Xu et al., 2002), a relationship potentially facilitating the stability of adhesive bonds by continuous dephosphorylation of β-catenin.

Several kinases have the potential to phosphorylate β-catenin, including Abl (Rhee et al., 2002), Src (Matsuyoshi et al., 1992; Hamaguchi et al., 1993; Behrens et al., 1993), the epidermal-growth-factor (EGF) receptor (EGFR) (Hoschuetzky et al., 1994; Ochiai et al., 1994; Kanai et al., 1995), and Fer (Rosato et al., 1998; Piedra et al., 2003), and therefore the potential to regulate the actin connection and cadherin function. Of these, only the non-receptor protein tyrosine kinase Fer (Greer, 2002) has been found associated with the cytoplasmic domain of N-cadherin (Arregui et al., 2001; Chen et al., 2003) and possibly E-cadherin (Kim and Wong, 1995; Rosato et al., 1998; Piedra et al., 2003). This association is mediated by binding to p120ctn (Kim and Wong, 1995), which in turn binds directly to a membrane proximal sequence of cadherin (Thoreson et al., 2000). The constitutive presence of Fer in association with the cytoplasmic domain of cadherin suggests that it does not routinely phosphorylate β-catenin and that it might be part of a mechanism indirectly regulating cadherin function through phosphorylation of cadherin-associated components. However, overexpression of Fer (Rosato et al., 1998; Piedra et al., 2003) or activation of Fer does result in phosphorylation of β-catenin at tyrosine 142, reducing the affinity of β-catenin for α-catenin (Piedra et al., 2003).

In this article, we demonstrate that one function of Fer is the phosphorylation of PTP1B on tyrosine 152, facilitating the binding of PTP1B to the cadherin cytoplasmic domain and stabilizing adhesions through dephosphorylation of β-catenin. To accomplish this, we used two cell systems in which the association between Fer and N-cadherin is disrupted: embryonic neural retina cells treated with peptides that block the Fer-p120ctn interaction; and embryonic fibroblasts from mice homozygous for a Fer-inactivating mutation, which results in destabilization and rapid degradation of Fer (Craig et al., 2001). In both cases, loss of Fer from the cadherin complex correlates with loss of cadherin-bound PTP1B, increased tyrosine phosphorylation of β-catenin at tyrosine 654 and loss of the cadherin/β-catenin interaction. Our results indicate that cadherin and β-catenin are maintained at cell-cell junctions by a mechanism dependent on Fer activity.

Materials and Methods

Antibodies

Anti-N-cadherin antibody NCD-2 (Hatta and Takeichi, 1986) was purified from hybridoma culture medium (Balsamo et al., 1991). Anti-pan-cadherin was from Sigma (St Louis, MO). Polyclonal anti-Fer antibody was prepared from a glutathione-*S*-transferase (GST) fusion peptide (Haigh et al., 1996). Anti-β-catenin antibodies used were from BD Transduction Labs (Lexington, KY) and a polyclonal anti-peptide antibody (Xu et al., 2002). Anti-phosphotyrosine (PY20), anti-p120ctn and anti-PTP1B were from BD Transduction Labs. Anti-PTP1B was also purchased from Calbiochem (San Diego, CA), as was anti-GST antibody. Anti-His-tag was from Novagen (Madison, WI). Horseradish-peroxidase (HRP) or alkaline phosphatase (AP)-conjugated secondary antibodies were from Organon Teknika (Durham, NC). Antibodies conjugated to magnetic beads, used in immunoprecipitation, were from Polysciences (Warrington, PA).

Preparation of Fer and p120ctn constructs

Fer and p120ctn constructs were generated using the polymerase chain reaction (PCR) and subcloned into PGEX-KG (Amersham Pharmacia Biotech). Full-length p120ctn was subcloned into pCAL-n-Flag (Stratagene, La Jolla, CA). The amino acids included in each fragment are shown in Fig. 1A and Fig. 2A. All clones were sequenced to ensure their fidelity and transformed into Epicurean coli BL21 (Stratagene, La Jolla, CA). Cultures were induced by 0.4 mM IPTG for 3 hours, harvested by centrifugation at 3000 ***g*** for 5 minutes and the pellets stored at –80°C until ready for use. The cell pellets were lysed in B-PER (Pierce) containing 1% bacterial protease inhibitor mixture (Sigma). GST fusion proteins were purified on glutathione/Sepharose-4B (Pharmacia Biotech, Piscataway, NJ). CBP-p120ctn was purified on calmodulin affinity resin (Stratagene). The expression of recombinant Fer and p120ctn peptides was confirmed by sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) and western blot. Hemagglutinin (HA)-tagged, full-length wild-type Fer was also subcloned into pcDNA3.1(+)zeo (Invitrogen, Carlsbad, CA) for mammalian expression.

Preparation of cell-permeable peptides

Oligonucleotides corresponding to the following peptides were synthesized by Integrated DNA Technologies (Iowa City, IA) with flanking restriction sites, and subcloned into TransVector (Q-BIOgene, Carlsbad, CA) in frame with the cDNA corresponding to the cell permeable *Antennapedia* fragment. Peptides were induced by 0.4 mM IPTG, purified on Ni-NTA agarose beads (Sigma) and analysed by SDS-PAGE on a 16.5% Tris-Tricine/peptide ready gels (Bio-Rad).

The peptide sequences used are as follows.

FerP: EKRIEESSETCEKKSDIVLLLSQKQALEEL

p120P: DGTTRRTETTVKKVVKTMTTRTVQPV

FerCo: LEELAQKQSLLLVIDSKKECTESSEEIRKE

P120Co: VPQVTRTTMTKVVKKVTTETRRTTGD

The elution buffer was replaced with sterile deionized water using a 3000MW Centricon (Millipore, Bedford MA), and the peptides stored in small aliquots at –70°C.

The cell-permeable peptide mimicking the p120ctn binding domain in N-cadherin (cad120P) was chemically synthesized by Genemed Synthesis (San Francisco, CA) and had the following sequence: DEEGGGEEDDYDLSQLQ. Control peptide consisted of the Antennapedia sequence only.

In vitro binding assay

Purified full-length CBP-p120ctn and GST-p120ctn constructs were biotinylated using EZ-link Sulfo-NHS-LC-Biotin (Pierce). Binding assays were carried out as previously described (Xu et al., 2002). Biotinylated p120ctn constructs were immobilized on NeutrAvidin-coated, clear strip 96-well plates (Pierce) and incubated with purified GST-Fer or GST-Fer-His. Bound Fer was determined by enzyme-linked immunosorbent assay (ELISA) using anti-GST or anti-His antibodies followed by HRP-conjugated secondary antibodies.

Peptide perturbation and immunoprecipitations

E7 retina cells were incubated in HBSGKCa (20 mM Hepes, pH 7.2, 150 mM NaCl, 3 mM KCl, 2 mM glucose, 1 mM $CaCl_2$) with the indicated peptides at a concentration of 10 μg ml^{-1}, at room temperature for 45 minutes, 1 hour or 2 hours. The cells were washed in PBS and lysed in mild lysis buffer [MLB; 1% NP-40 in PBS containing protease inhibitor cocktail (Roche) and 1 mM sodium orthovanadate] at 4°C for 30 minutes. The lysates were cleared at 15,000 ***g*** for 10 minutes and aliquots containing equivalent amounts of protein were incubated overnight with NCD-2 at 4°C with mixing. Goat anti-rat-antibody-conjugated magnetic beads (50 μl) were then

added and the mixture incubated at 4°C for 1 hour. The magnetic beads were collected using a magnetic stand, washed three times with lysis buffer and once with PBS, dissolved in SDS sample buffer, fractioned by SDS-PAGE, transferred to PVDF membranes and immunoblotted with anti-Fer, anti-p120, anti-β-catenin or anti-PTP1B antibodies. To determine phosphorylation of β-catenin, cells were lysed in RIPA buffer (PBS containing 0.1% SDS, 0.1% sodium deoxycholate, 1% NP-40, 50 mM NaF, 1 mM sodium orthovanadate and protease inhibitor cocktail). The cleared lysates were immunoprecipitated with anti-β-catenin antibody and immunoblotted with PY20. For immunoprecipitation of wild-type or Fer D743R fibroblasts, confluent cultures were washed in HBSGKCa and lysed in buffer containing 0.5% NP-40, 20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM NaF, 1 mM sodium orthovanadate and protease inhibitor cocktail. The lysates were centrifuged at 14,000 ***g*** for 10 minutes and the supernatants incubated with 1 μl rabbit IgG and 20 μl Pansorbin (Calbiochem) for 30 minutes. The supernatants were then incubated with anti-pan-cadherin antibody for 2-4 hours at 4°C, followed by protein-G-conjugated magnetic beads (Dynal, Lake Success, NY) and processed as described above.

To determine cell surface expression of N-cadherin, retina cells treated with peptides were incubated with NHS-PEO4-Biotin (Pierce), washed extensively and lysed in RIPA buffer. Biotinylated proteins were bound to avidin-agarose beads (Pierce), the beads were washed extensively and bound proteins fractionated by SDS-PAGE and assayed by western blots with anti-N-cadherin antibody NCD-2.

Introduction of exogenous β-catenin into cells

To force the expression of exogenous β-catenin, E8 retina cells prepared by trypsin dissociation (Balsamo et al., 1991) were incubated with BioPorter (GTS, San Diego, CA) and the indicated GST fusion peptide for 3 hours at room temperature, followed by 1 hour with added FerP or FerCo. Cells were then collected, lysed in MLB and immunoprecipitated with anti-N-cadherin antibody as described above. Alternatively, cells were aliquoted into wells precoated with Fc-N-cad [chick N-cadherin ectodomain fused with the Fc fragment of mouse IgG2b (Lambert et al., 2000)] and assayed for adhesion as described below.

Cell adhesion and neurite outgrowth assays

96-well plates precoated with mouse anti-IgG (Bio-Coat; BD Biosciences, Billerica, MA) were incubated with purified Fc-N-cad diluted in PBS containing 2% bovine serum albumen (BSA). Alternatively, wells were coated with poly-L-lysine (50 μg ml^{-1} in PBS) followed by laminin (40 μg ml^{-1} in PBS). The wells were washed with PBS and blocked with 2% BSA for 1 hour. E8 chicken neural retina cells were prepared by trypsin dissociation in the presence of calcium (Balsamo et al., 1991) and incubated with cell-permeable peptides. After 45 minutes, cells were aliquoted onto the treated wells and incubated for 1 hour, non-adherent cells were washed and bound cells were quantified using crystal violet (Balsamo et al., 1998).

Substrates for neurite growth were prepared by coating eight-well slides with poly-lysine followed by NCD-2 or laminin, followed by washing and blocking with 2% BSA. The presence of neurites was quantitatively assessed in sparse single cell cultures of E8 chick neural retina. Peptides were added at 10 μM 2 hours after plating. After overnight culture in DMEM containing 1% ITS (insulin-transferin-selenium; Gibco-BRL, Grand Island, NY) cells were fixed in 4% *p*-formaldehyde and ~200 cells were evaluated for the presence of neurites. Neurite growth was visualized using phase optics.

For aggregation assays, wild-type or D743R Fer fibroblasts grown to near confluence on 100 mm plates were washed in PBS and released from the plate with 0.025% Trypsin (Gibco-BRL) in PBS. Cells were washed in DMEM containing 5 μg ml^{-1} Antipain and 10 μg ml^{-1} DNase (Sigma-Aldrich) and added to 35 mm dishes containing 3 ml DMEM buffered with Hepes pH 7.2 with or without 5 mM EGTA. Cultures were incubated at 37°C for 3 hours with shaking at 80 rpm and then visualized using an Axiovert 25 microscope equipped with a CCD camera (Zeiss).

Immunocytochemistry

Cells grown on eight-well slides (Falcon) were fixed in 4% *p*-formaldehyde prepared in cytoskeletal buffer (10 mM Mes pH 6.1, 138 mM KCl, 3 mM $MgCl_2$, 2 mM EGTA) and permeabilized in 0.5% Triton X-100 for 10 minutes at room temperature. Cells were then blocked with 3% BSA in TBS for 1 hour and incubated with the primary antibody diluted in 1% BSA in TBS for 1 hour at room temperature, followed by the appropriate Alexa-488-labeled secondary antibody (Molecular Probes) diluted in 1% BSA in TBS. After 1 hour, the cells were washed, incubated with Alexa-568-labeled phalloidin (Molecular Probes) for 20 minutes at room temperature, mounted in Vectashield (Vector Labs) and observed by confocal microscopy.

In vitro phosphorylation of PTP1B

Purified phosphatase-dead (C215S) GST-PTP1B carrying additional mutations on tyrosine 66 (C215S/Y66F), tyrosine 152 (C215S/Y152F) or both tyrosine 66 and tyrosine 152 (C215S/Y66/152F) was immobilized on glutathione-coated wells and incubated with recombinant wild-type Fer (FerWT), kinase-dead Fer (FerD743R) or FerWT in the presence of genistein, and the amount of phosphotyrosine incorporated into PTP1B was evaluated by ELISA using HRP-conjugated anti-phosphotyrosine antibody. Alternatively, immobilized GST-PTP1B was incubated with Fer, eluted with SDS sample buffer and the amount of phosphotyrosine evaluated by western blot with anti-phosphotyrosine antibody.

Results

Mapping the domain on Fer and p120ctn essential for interaction

The interaction of Fer and p120ctn was previously mapped to the 400 N-terminal residues in Fer (Kim and Wong, 1995). To be able to specifically disrupt Fer binding to p120ctn and thus potentially inhibit the association of Fer with the N-cadherin complex of proteins, we sought to identify more narrowly the amino acid sequences involved in the Fer/p120ctn interaction. Full-length p120ctn was expressed as a calmodulin-binding protein (CBP) fusion peptide, purified by calmodulin affinity chromatography, biotinylated and immobilized on streptavidin-coated wells. GST-Fer constructs were then added to the wells at increasing concentrations and binding was determined using an anti-GST antibody. A diagram of the GST-Fer peptides used is shown in Fig. 1A. The Fer construct containing the N-terminal coiled-coil domains CC1, CC2 and CC3 (Fer-2) binds to p120ctn as well as the full-length GST-Fer (Fer-1). By contrast, Fer-4, which consists of the SH2 and kinase domains, shows no significant interaction with p120ctn (Fig. 1B). Further fragmentation of the N-terminal region narrowed the binding domain to a sequence between residues 305 and 380 (Fer-3), corresponding to CC2 and CC3 (Fig. 1B). We further divided Fer-3 into three smaller peptides, Fer-5, Fer-6 and Fer-7 (Fig. 1A). Of these, only Fer-7 retains the ability to bind to p120ctn (Fig. 1B) as well as full-length GST-Fer (Fig. 1C). Fer-7 corresponds to residues 331-360, comprising the C-terminal region of CC2 and the N-terminal region of CC3 (Fig. 1A).

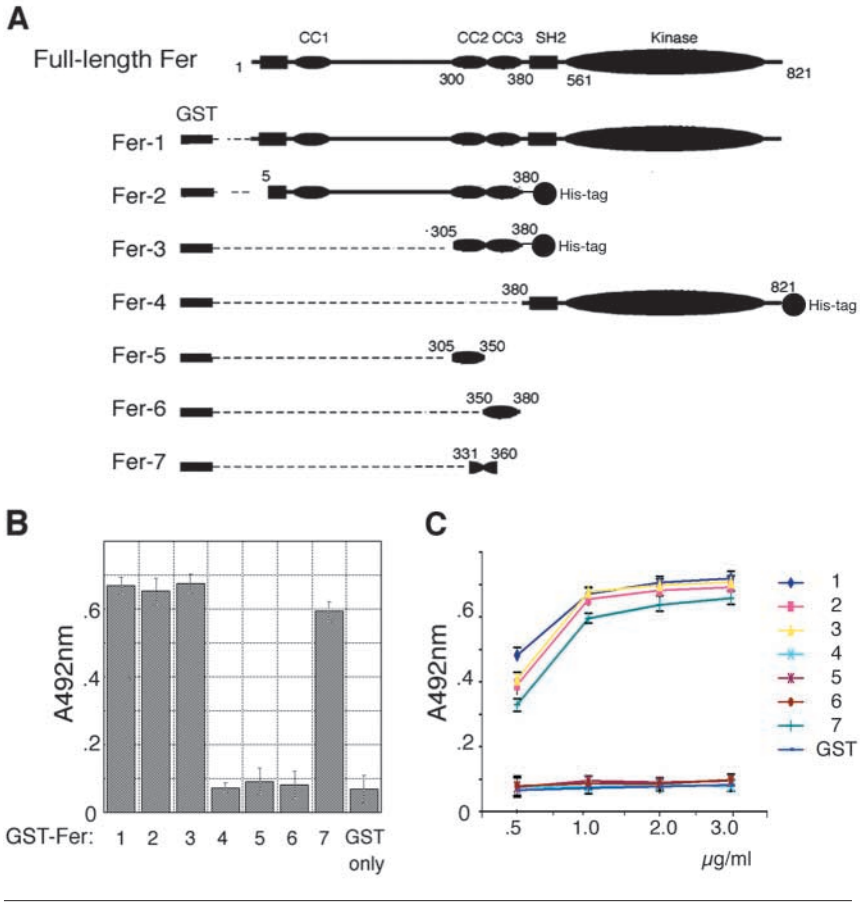

Fig. 1. Binding of Fer to p120ctn. (A) Full-length and GST fusion constructs of Fer used in the in vitro binding assay. CC1, CC2 and CC3 correspond to coiled-coil domains. (B) Recombinant GST-Fer peptides were purified by affinity chromatography on glutathione-agarose and incubated with biotin-conjugated CBP-p120ctn immobilized on streptavidin-coated wells. Bound protein was determined by ELISA using anti-GST antibody and the appropriate HRP-conjugated secondary antibody. (C) Dose response of binding of the GST-Fer constructs to immobilized p120ctn. Immobilized p120ctn was incubated with increasing concentrations of GST-Fer constructs 1-7 and bound protein determined as described in B.

We used a similar strategy to analyse the domain in p120ctn that is responsible for interaction with Fer. In this case, GST-p120ctn was biotinylated and immobilized on streptavidin-coated wells and binding to His-tagged Fer-3 was determined using an anti-His-tag antibody. A diagram of the p120ctn constructs used is shown in Fig. 2A. We began by preparing an N- and a C-terminal fragment of roughly equal size. Binding is limited to the N-terminal 500 residues (P120-1, Fig. 2B). Further analysis allowed us to define a 26-amino-acid region fragment P120-10 (amino acid residues 131-156) able to bind to Fer-3 as well as the N-terminal fragment of p120ctn P120-1 (Fig. 2A-C).

Loss of Fer results in disruption of the N-cadherin complex of proteins and loss of cadherin function

To disrupt the interaction of Fer with the N-cadherin complex, we designed two cell-permeable peptides corresponding to Fer-7 (residues 331-360; FerP) and P120-10 (residues 131-156; p120P) covalently linked to the Antennapedia-derived penetratin peptide (Derossi et al., 1994; Prochiantz, 1996). These peptides compete for the

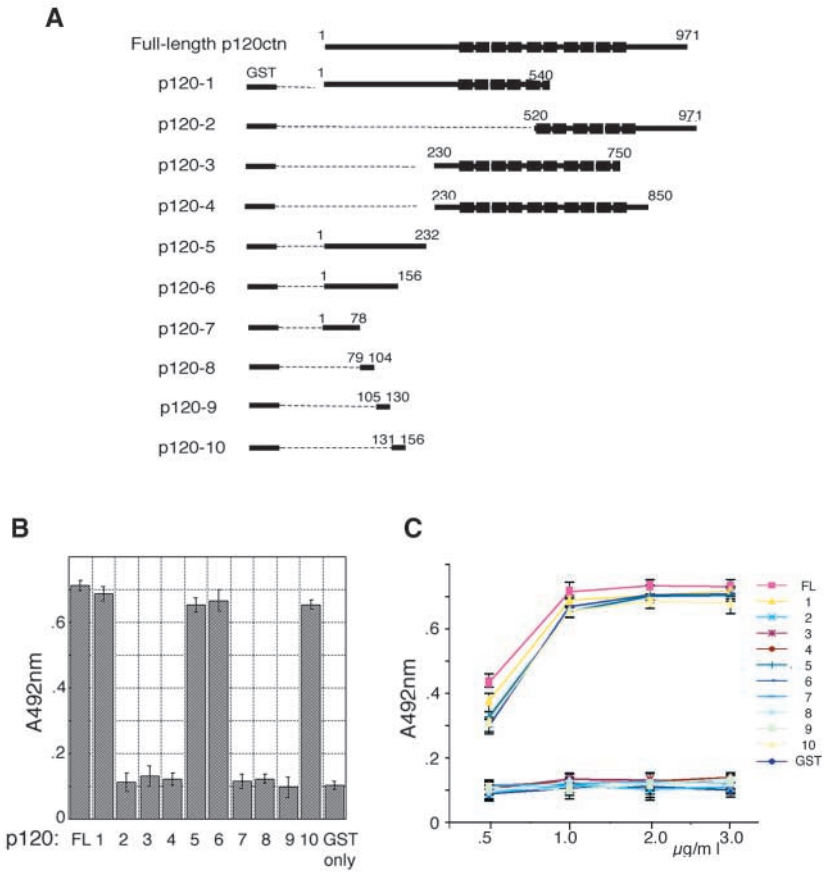

Fig. 2. Binding of p120ctn to Fer. (A) Full-length and GST constructs of p120ctn used in the in vitro binding assay. Filled rectangles represent the Armadillo domains. (B) Recombinant GST-p120ctn peptides conjugated to biotin were immobilized on streptavidin-coated wells and incubated with His-tagged Fer-3. Bound protein was determined by ELISA using anti-His-tag antibody and HRP-conjugated second antibody. (C) Dose response of binding of the biotinylated GST-p120ctn peptides to His-Fer-3. His-Fer-3 was incubated with increasing concentrations of immobilized GST-p120ctn constructs 1 to 10, and bound protein was determined as described in B.

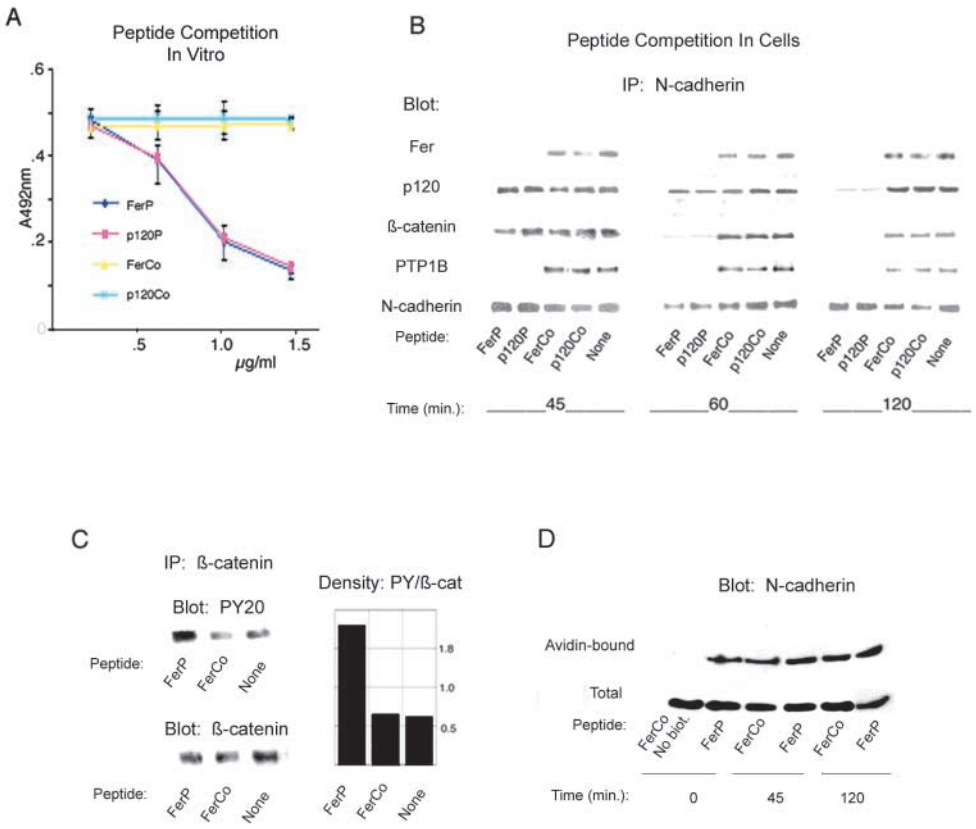

Fig. 3. Effect of cell-permeable Antennapedia fusion peptides that mimic the Fer-p120ctn binding site on in vitro binding between Fer and p120ctn, and on the composition of the cadherin complex of proteins in E8 chick retina cells. (A) Biotin-labeled CBP-p120ctn was immobilized on streptavidin wells and incubated with 3 μg GST-Fer per well in the presence of increasing concentrations of competing peptides. Bound Fer was estimated using anti-GST antibody. Abbreviations: FerP and FerCo, Antennapedia cell-permeable peptides covalently attached to the Fer-7 or the reverse Fer-7 sequence, respectively; p120P and p120Co, Antennapedia peptides covalently attached to the P10 or the reverse P10 sequence. (B) Intact E8 retina cells were incubated in the presence of the indicated peptide for 45 minutes, 60 minutes or 120 minutes at room temperature. The cells were homogenized in buffer containing 1% NP-40 and immunoprecipitated with NCD-2. The resulting immunocomplexes were analysed by western blot with the indicated antibody. (C) Intact E8 retina cells were incubated in the presence of the indicated peptide for 60 minutes at room temperature, lysed in RIPA buffer, immunoprecipitated with anti-β-catenin antibody and analysed by western blot using anti-phosphotyrosine antibody. The blots were then stripped and blotted with anti-β-catenin antibody. The relative intensity of the β-catenin western blot bands was measured by densitometry and is presented as the ratio between phosphotyrosine β-catenin and total β-catenin. (D) Retina cells were incubated with the indicated peptides and labeled with a cell-impermeable biotinylation reagent for the indicated time. The cells were lysed as above, cell-surface N-cadherin pulled down with avidin-coated beads, fractionated by SDS-PAGE and visualized by western blot with anti-N-cadherin antibody. An aliquot of the total lysate was also analyzed by western blot, for comparison.

interaction between p120ctn and Fer in vitro (Fig. 3A) and thus have the potential to disrupt the same interaction in intact cells. When E8 retina cells bearing functional cell-surface N-cadherin are incubated for 45 min with FerP, p120P, but not control peptides consisting of the reverse FerP or p120P sequences (FerCo and p120Co, respectively), Fer is lost from the N-cadherin complex (Fig. 3B). Coincident with the loss of Fer from the N-cadherin complex, PTP1B is also lost (Fig. 3B), and increasing the time of incubation of cells with FerP or p120P results in loss of β-catenin after 1 hour and p120ctn after 2 hours (Fig. 3B). During this period, the total amount of these components is altered minimally (not shown). These data indicate that the peptides cause a progressive disruption of the N-cadherin complex of proteins, initiated by the loss of Fer and PTP1B, followed closely by β-catenin and ultimately p120ctn. Furthermore, concomitant with the loss of β-catenin, its phosphorylation on tyrosine residues is increased at least threefold, as reflected in the ratio of phosphorylated β-catenin to total β-catenin obtained by scanning immunoblots following two different exposures (Fig. 3C).

Consistent with the progressive disruption of the cadherin complex of proteins, disruption of the Fer-p120ctn interaction using FerP or p120P results in loss of N-cadherin-mediated cell adhesion: a minimum of 60% reduction is observed on incubation of cells with FerP or p120P for 1 hour, whereas the same concentrations of FerCo or p120Co have no effect (Fig. 4A). This reduction is not due to a loss of N-cadherin from the cell surface; comparison of biotinylated cadherin on intact cells at 0 minutes, 45 minutes and 2 hours of incubation in peptide reveals no overt change in the amount of cadherin (Fig. 3D). Incubation in the presence of FerP also impairs β1-integrin-mediated adhesion among E8 neural retina cells (Fig. 4B). This is in agreement with our previous results demonstrating that loss of Fer from the N-cadherin complex results in translocation of Fer to the β1-integrin complex, and consequent loss of β1-integrin adhesion function (Arregui et al., 2000; Lilien et al., 1999).

We also assayed the effect of FerP and p120P on neurite extension. Dissociated E7 cells were plated for 2 hours in eight-well slides coated with laminin or NCD-2 and further incubated for 16 hours in the presence of FerCo or FerP. The cells were then visualized under phase contrast and the proportion of single cells bearing neurites longer than two cell diameters was evaluated. Neurite growth is greatly reduced in cells treated with FerP, both on N-cadherin and laminin substrates (Fig. 4C). In the presence of FerP and p120P some cellular extensions persist

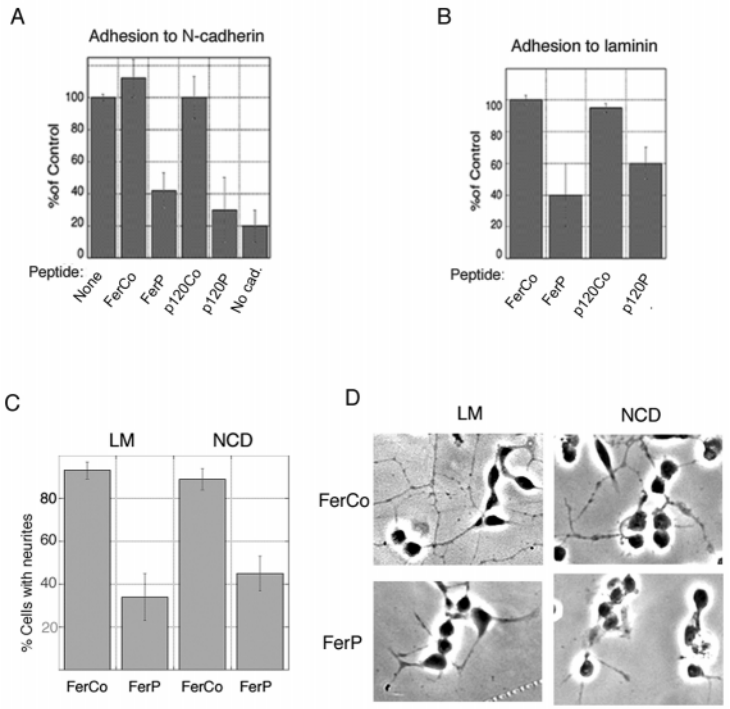

Fig. 4. Effect of cell permeable Antennapedia fusion peptides that mimic the Fer-p120ctn binding site on cell adhesion and neurite outgrowth. (A,B) Adhesion of E8 chick retina cells to cadherin (A) and laminin (B). Dissociated E8 chick neural retina cells were incubated for 1 hour at room temperature in the presence of the indicated peptide. Equal numbers of cells were then aliquoted into wells precoated with the extracellular domain of N-cadherin, Fc-N-cad (A) or with laminin (B) and incubated for 1 hour in the presence of the indicated peptides. Adherent cells were stained with crystal violet and quantified by absorbance at 560 nm. No adhesion is observed onto uncoated, blocked wells (No Ncad). Adhesion is expressed as the percentage of adhesion in the absence of added peptides (A) or in the presence of control peptide FerCo (B). (C,D) Neurite outgrowth. E7 chick retina cells were plated on slides coated with anti-N-cadherin antibody NCD-2 or laminin. Single cells bearing neurites longer than two cell diameters were counted and expressed as a percentage of the total adherent cells (C). Cells and neurites were visualized under phase contrast (D).

or develop; however, these appear thicker and do not extend beyond two or three cell diameters (Fig. 4D).

To compare the effects of loss of Fer to loss of p120ctn and associated Fer, we used a cell-permeable peptide that mimics the p120ctn target binding sequence in cadherin (Thoreson et al., 2000). Incubation of retina cells in this peptide for 1 hour results in loss of p120ctn and Fer, as well as β-catenin and PTP1B, from the cadherin complex (Fig. 5A), with a concomitant increase in the pool of tyrosine phosphorylated β-catenin (Fig. 5B). As with FerP, the total amounts of these components are altered minimally (not shown). Cad120P, like FerP, also results in a loss of cadherin-mediated adhesion, in spite of the fact that the amount of cell surface cadherin remains constant during this time (Fig. 5C,D). Thus, during the 2-hour course of these experiments, there is no substantial difference between the selective competitive removal of Fer from its association with p120ctn and the selective competitive removal of p120ctn and associated Fer.

Fer phosphorylates PTP1B on tyrosine 152

The loss of PTP1B concomitant with Fer (Fig. 3B) and the requirement for PTP1B phosphorylation on tyrosine 152 to bind to cadherin (Rhee et al., 2001) infers that PTP1B might be a direct substrate of Fer. The consensus target phosphorylation site for enzymes of the Fes/Fer family was identified as tyrosine followed by two hydrophilic residues and valine or isoleucine (Songyang et al., 1994). Tyr152 is present in just such a consensus (YYTV). To determine whether Tyr152 in PTP1B is a substrate for Fer, we created a GST-PTP1B fusion protein lacking phosphatase activity (C215S) and carrying the mutation Y152F or Y66F, and assayed them for in vitro phosphorylation by recombinant Fer. Recombinant PTP1Bs were incubated with recombinant wild-type Fer, with a catalytically inactive D743R mutant Fer, with wild-type Fer in the presence of genistein (a tyrosine kinase inhibitor) or in the absence of Fer in buffer containing ATP as a source of phosphate groups. Wild-type Fer can phosphorylate PTP1B in vitro and a mutation at tyrosine 66 has no effect on phosphorylation, whereas a mutation at tyrosine 152 completely abolishes phosphorylation (Fig. 6). No in vitro phosphorylation is observed with catalytically inactive Fer (D743R), wild-type Fer in the presence of genistein, or in the absence of Fer (Fig. 6).

Introduction of β-catenin with a Y654F mutation into cells prevents the loss of cadherin-mediated adhesion due to FerP

The loss of β-catenin from the cadherin complex as a result of loss of Fer, coupled with its increased phosphorylation, suggests that β-catenin is a downstream target of a signal initiated by Fer. Because phosphorylation of tyrosine 654 in β-catenin has been implicated in the association of β-catenin with cadherin (Piedra et al., 2001), we hypothesized that, in cells expressing a β-catenin mutant that cannot be phosphorylated on tyrosine 654, uncoupling Fer from the cadherin complex would have no effect on cadherin function.

To test this hypothesis, we prepared wild-type GST/β-catenin fusion proteins as well as GST fusions to β-catenin carrying a Y654F mutation or a Y142F mutation. Phosphorylation of Y142 has been implicated in regulating the association of β-catenin with α-catenin but has no effect on the association between β-catenin and cadherin (Piedra et al., 2003). The GST fusion proteins were introduced into embryonic neural retina cells using BioPorter. This reagent is very effective at introducing intact proteins into retinal cells: more than 70% of the cells are transfected using BioPorter (not shown). Cells were then incubated with FerP or CoP (see above) and lysed in neutral detergent. The lysates were immunoprecipitated with anti-cadherin antibody, fractionated by SDS-PAGE, transferred to PVDF and immunoblotted with anti-β-catenin antibody. Cells preloaded with the GST/wild-type or GST/mutant-β-catenin showed two bands that were immunoreactive with anti-β-catenin antibody: one at approximately 92 kDa, corresponding to endogenous β-catenin, and a higher-molecular-weight band corresponding to

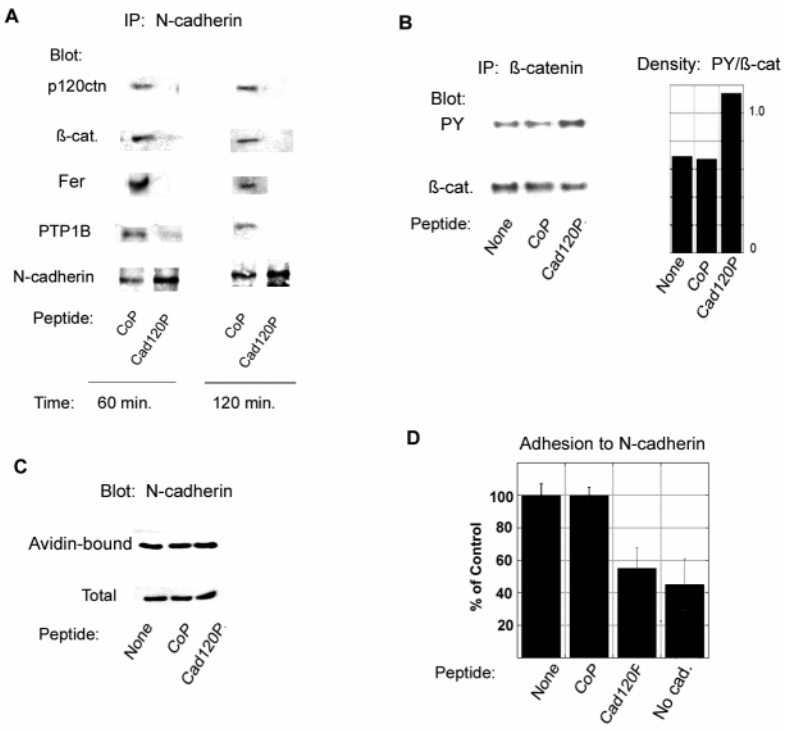

Fig. 5. Effect of cell permeable Antennapedia fusion peptides that mimic the p120ctn binding site on N-cadherin on the composition and function of the cadherin complex of proteins in E9 chick retina cells. (A) Intact E9 retina cells were incubated in the presence of the indicated peptide for 60 minutes or 120 minutes at room temperature. The cells were homogenized in buffer containing 1% NP-40 and immunoprecipitated with NCD-2. The resulting immuno-complexes were analyzed by western blot with the indicated antibody. (B) Intact E9 retina cells were incubated in the presence of the indicated peptide for 60 minutes at room temperature lysed in RIPA buffer, immunoprecipitated with anti-β-catenin antibody and analysed by western blot using anti-phosphotyrosine antibody. The blots were then stripped and blotted with anti-β-catenin antibody. (C) Retina cells were incubated with the indicated peptides and labeled with a cell-impermeable biotinylation reagent. The cells were lysed as above, cell-surface N-cadherin 'pulled down' with avidin-coated beads, fractionated by SDS-PAGE and visualized by western blot with anti-N-cadherin antibody. An aliquot of the total lysate was also analyzed by western blot, for comparison. (D) Dissociated E8 chick neural retina cells were incubated for 1 hour at room temperature in the presence of the indicated peptide. Adhesion to immobilized Fc-N-cad is expressed as the percentage of adhesion in the absence of added peptide.

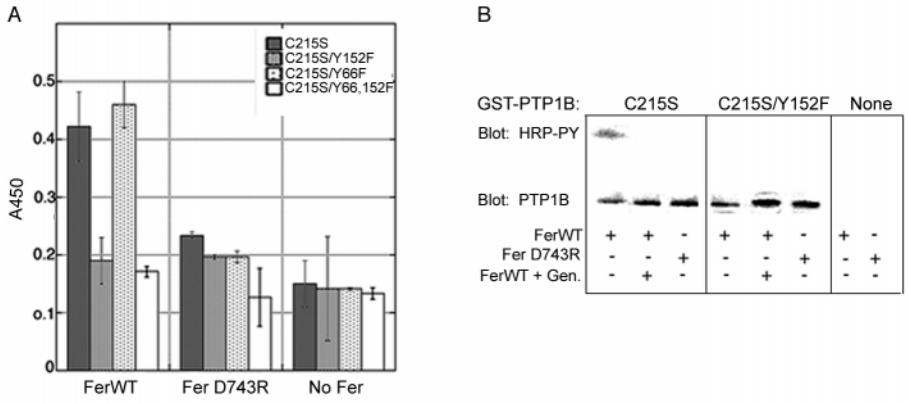

Fig. 6. In vitro phosphorylation of PTP1B by Fer. Purified, phosphatase-dead (C215S) GST-PTP1B was immobilized on glutathione-coated wells and incubated with recombinant wild-type Fer (FerWT) or kinase-dead Fer (FerD743R), and the amount of phosphotyrosine incorporated into PTP1B was measured using anti-phosphotyrosine antibodies. (A) PTP1B carrying the C215S mutation and additional mutations on tyrosine 66 (C215S/Y66F), tyrosine 152 (C215S/Y152F) or both tyrosine 66 and 152 (C215S/Y66/152F) was used as substrate and phosphorylation evaluated by ELISA using HRP-conjugated anti-phosphotyrosine antibody. (B) Immobilized kinase dead GST-PTP1B was incubated with Fer, eluted with SDS sample buffer and the amount of phosphotyrosine evaluated by western blot with anti-phosphotyrosine antibody (top). The same membrane was stripped and reprobed with anti-PTP1B antibody (bottom).

the β-catenin/GST fusion. Densitometric comparison of endogenous and introduced wild-type β-catenin indicates that close to 50% of the cadherin is associated with exogenously introduced β-catenin (not shown). After treatment of cells with FerP, the GST/wild-type-β-catenin and GST/Y142F-β-catenin are no longer detected in complex with N-cadherin (Fig. 7A). By contrast, the GST/Y654F-β-catenin remains associated with N-cadherin after the loss of Fer, indicating that tyrosine 654 is indeed the target of a Fer-mediated signal cascade (Fig. 7A). Endogenous β-catenin is lost from the cadherin complex after FerP treatment in all cell types, as expected (Fig. 7A).

The effect of FerP on cadherin-mediated adhesion among cells expressing the GST/β-catenin fusion proteins was also tested. Adhesion is lost in cells expressing GST/wild-type-β-catenin or GST/Y142F-β-catenin after treatment with FerP (Fig. 7B). However, among cells expressing the mutant Y654F, the effect of FerP is much reduced (Fig. 7B) further implicating β-catenin tyrosine residue 654 as a target of the Fer-mediated signal cascade.

Loss of catalytically active Fer results in loss of PTP1B associated with the cadherin complex

The results presented so far suggest that dissociation of Fer from the N-cadherin complex of proteins leads to loss of N-cadherin-associated PTP1B. This, in turn, compromises the ability to dephosphorylate β-catenin with retention of phosphate on tyrosine residue 654, resulting in uncoupling of β-catenin from N-cadherin and consequent loss of N-cadherin-mediated adhesion and neurite outgrowth. To explore further

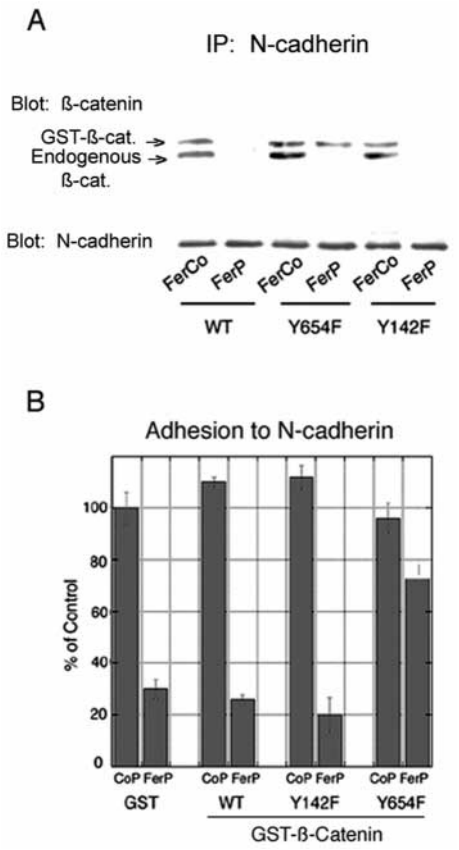

Fig. 7. Effect of FerP on cells expressing mutant β-catenin. GST, GST/wild-type-β-catenin, GST/Y142F-β-catenin or GST/Y654F-β-catenin were introduced into E8 chick neural retina cells using BioPorter and the cells treated with FerP or FerCo peptides for 1 hour. (A) Cells were lysed in nonionic detergent, immunoprecipitated with anti-cadherin antibody and immunoblotted with the indicated antibody. (B) Intact cells were aliquoted into wells precoated with Fc-N-cad and assayed for adhesion. Adhesion is expressed as the percentage of adhesion among cells treated with GST and CoP.

the role of Fer in cadherin function, we prepared immortalized embryonic fibroblasts derived from mice homozygous for *fer*^{D743R}, a mutation that abolishes Fer kinase activity and leads to rapid degradation of the Fer protein (Craig et al., 2001). Indeed, using an antibody specific to Fer, we are unable to detect the 94 kDa Fer protein in total cell lysates of D743RFer fibroblasts (Fig. 8A). We also analysed the expression levels of cadherin, β-catenin, p120ctn and PTP1B (Fig. 8A). There is no detectable difference in cadherin or PTP1B expression between wild-type (WT) and mutant D743R fibroblasts, and a small but reproducible decrease in the amounts of β-catenin and p120ctn detected on immunoblots of D743R mutant cell lysates (Fig. 8A).

Immortalized fibroblasts from *fer*^{D743R} mice show that loss of Fer is correlated with disruption of β-catenin and PTP1B association with cadherin; immunoprecipitation with an anti-pan-cadherin antibody shows that the amount of β-catenin and PTP1B associated with cadherin in D743R fibroblast cell lysates is much reduced as compared with wild-type cells (Fig. 8B). This loss correlates with a detectable increase in phosphorylated tyrosine residues on β-catenin and a decrease in phosphotyrosine in PTP1B, as determined by immunoblot (Fig. 8B) and densitometric analysis (not shown).

Cadherin and β-catenin do not co-localize to cell-cell junctions in the absence of Fer

The loss of β-catenin and PTP1B from the cadherin complex in fibroblasts from *fer*^{D743R} mice led us to look at the distribution of those molecules in intact cells. Immortalized fibroblasts derived from wild-type or *fer*^{D743R} mouse embryos cultured to semi-confluence form a dense network of actin stress fibers (Fig. 8C). The distribution of cadherin and β-catenin, however, differ dramatically in the two cell types. Cells expressing wild-type Fer show cadherin and β-catenin present at points of cell-cell contact, forming classic zipper-like structures (Fig. 8C WT, arrowhead). By contrast, among fibroblasts from *fer*^{D743R} mice, cadherin and β-catenin (Fig. 8C) are either not visible or result in very weak staining at boundaries between apposing cells. Other adherens junction proteins such as vinculin and nectins are also not present at boundaries between cells (not shown).

Consistent with the lack of cadherin and β-catenin at boundaries between cells, D743R Fer fibroblasts are also unable to form Ca^{2+}-dependent adhesions. Visual or quantitative analysis of single cells prepared by trypsinization and cultured for 3 hours in suspension under rotation reveals that D743R Fer cells remain as single cells or form very small, labile cell clusters of two to three cells, whereas wild-type cells form large aggregates (Fig. 8D).

The mutant phenotype can be rescued by expression of wild-type Fer. Stably transfected cell lines were created expressing wild-type Fer cDNA and tested for Fer expression by western blots with anti-Fer antibody (Fig. 9A). Following reintroduction of active Fer in D743R cells, β-catenin and PTP1B are again found associated with cadherin (Fig. 9B) and co-localization of cadherin and β-catenin to areas of cell contact is restored (Fig. 9C). Interestingly, and in agreement with previously published data (Rosato et al., 1998), transiently transfected D743R cells expressing high levels of wild-type Fer do not localize cadherin to cell-cell junctions (not shown), suggesting that Fer activity in the cadherin complex must be tightly regulated. Furthermore, forced expression of Y654F β-catenin, but not wild-type or Y142F β-catenin, in D743R Fer fibroblasts also rescues the mutant phenotype, restoring the co-immunoprecipitation of β-catenin with cadherin (Fig. 9D) and the ability of cells to form calcium-dependent aggregates (Fig. 9E, D743R/Y654F). Thus, as in retinal cells, it is tyrosine 654 of β-catenin that is hyperphosphorylated when Fer is absent from the cadherin complex.

EGF rescues the *fer*^{D743R} phenotype

Mice carrying the *fer*^{D743R} mutation have not been extensively analysed. However, the amount of β-catenin and PTP1B associated with N-cadherin in tissue samples is reduced by approximately 50% in comparison to controls (Fig. 10A), suggesting that N-cadherin-mediated adhesions might be compromised, a possibility we are testing. In spite of this defect and the marked change in cadherin/β-catenin localization and physical association seen in D743R immortalized fibroblasts, *fer*^{D743R} mutant mice are viable and fertile (Craig et al., 2001;

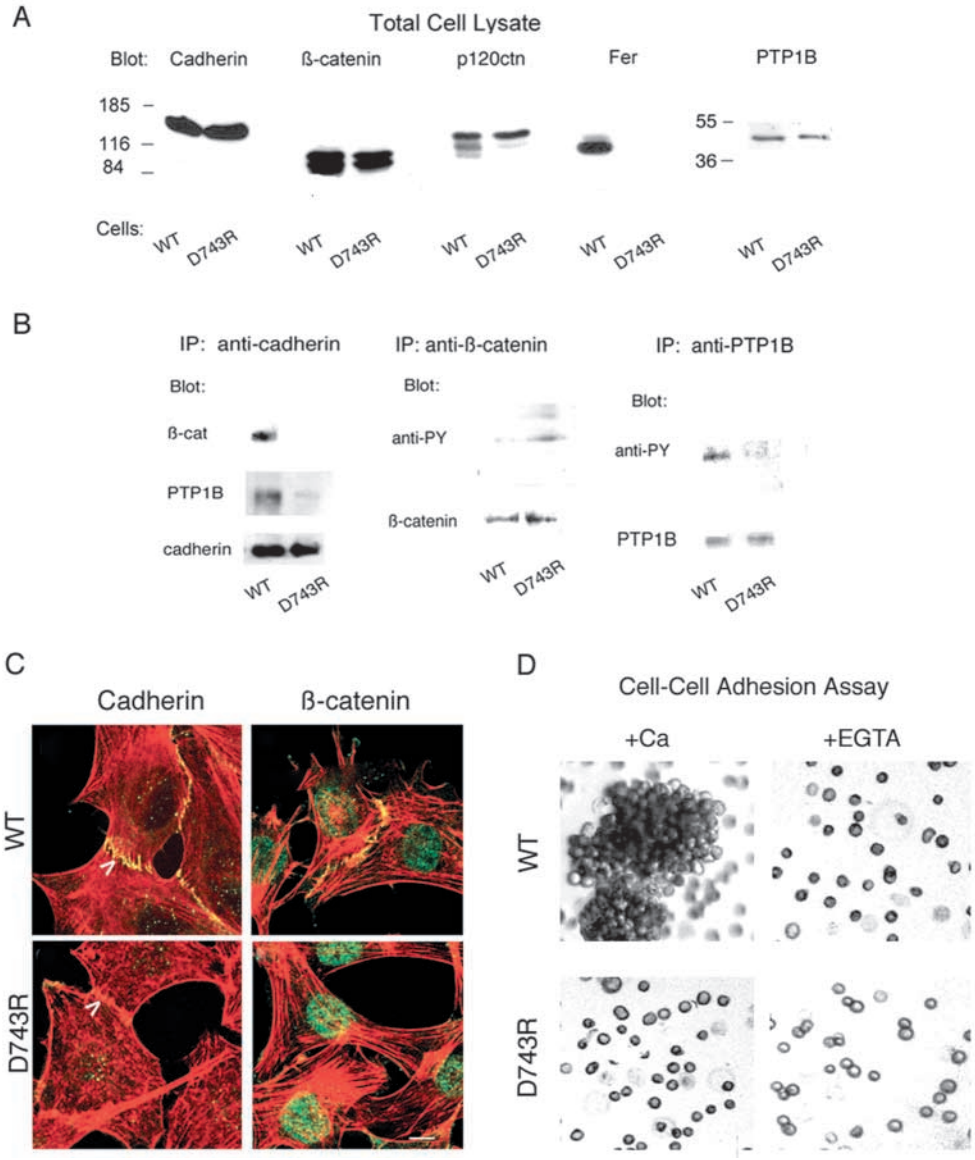

Fig. 8. Expression and localization of N-cadherin and associated proteins among immortalized fibroblasts derived from wild-type and *fer*^{D743R} mice. Fibroblasts derived from wild-type (WT) or *fer*^{D743R} (D743R) embryos were cultured to confluence, washed free of serum and lysed in mild detergent buffer. (A) Equal amounts of protein from cell lysates were analysed by western blot using the indicated antibodies. (B) Equal amounts of protein were immunoprecipitated with anti-pan-cadherin, anti-β-catenin or anti-PTP1B antibodies and the immunoprecipitates analysed by western blot using the indicated antibodies. (C) WT or D743R cells were stained with Alexa-568/phalloidin and anti-pan-cadherin or anti-β-catenin antibodies, as indicated, followed by the appropriate Alexa-488-conjugated second antibody, and visualized by confocal microscopy. Among WT cells, cadherin 'zippers' are clearly seen at cell boundaries, where actin and cadherin or actin and β-catenin overlap (arrowhead). In D743R cells, there is no detectable cadherin or β-catenin at cell boundaries (arrowhead). Scale bar, ~10 μm. (D) Aggregation of WT and D743R cells in suspension cultures. Single cells from WT or D743R cultures were prepared by trypsin dissociation and allowed to aggregate in the presence of 1 mM Ca^{2+} or 5 mM EGTA for 3 hours at 37°C and with shaking at 80 rpm, in 30 mm dishes. Cells were visualized under phase-contrast and photographed using an Axiovert25 microscope and Axiovision system (Zeiss).

Greer, 2002). The lack of cell surface cadherin among D743R cells in vitro is not due to immortalization, because primary fibroblasts from *ferD743R* mice are also unable to efficiently localize cadherin at points of cell-cell contact (not shown). This suggests that a compensatory mechanism exists in the mutant mice, attenuating the need for Fer to target PTP1B to the cadherin complex. Indeed, stimulation of D743R cells in vitro with EGF results in co-precipitation of β-catenin and PTP1B with cadherin (Fig. 10B) and a reduction in the amount of β-catenin phosphorylated on tyrosine residues (Fig. 10C). Consistent with this, EGF restores the ability to localize cadherin and β-catenin at cell surface (Fig. 10D). This suggests that during embryogenesis EGF, and possibly other growth factors, might function directly to phosphorylate PTP1B (Liu and Chernoff, 1997) or indirectly to activate another kinase, which in turn phosphorylates PTP1B.

Discussion

The cadherin-actin connection, and thus cadherin function, is regulated through distinct tyrosine phosphorylation states of β-catenin. Phosphorylation at tyrosine 654 reduces the affinity of β-catenin for cadherin (Piedra et al., 2001) and disrupts adhesion and neurite outgrowth (Balsamo et al., 1996; Balsamo et al., 1998; Xu et al., 2002). In this article, we show that the tyrosine kinase Fer and tyrosine phosphatase PTP1B co-operate to regulate phosphorylation of tyrosine 654 in β-catenin, thus defining a new regulatory function for Fer and an epigenetic regulatory loop controlling cadherin function.

Fer is constitutively associated with the cytoplasmic domain of N-cadherin (Arregui et al., 2000; Chen et al., 2003) and E-cadherin (Kim and Wong, 1995; Rosato et al., 1998; Piedra et al., 2003). This association is mediated by binding to p120ctn (Kim and Wong, 1995), which in turn binds directly to a membrane proximal sequence of cadherin (Thoreson et al., 2000). We have shown that the non-receptor tyrosine phosphatase PTP1B is also constitutively associated with the N-cadherin cytoplasmic domain and that binding requires phosphorylation at tyrosine 152 (Rhee et al., 2001), the residue phosphorylated by Fer. Displacing bound PTP1B with a

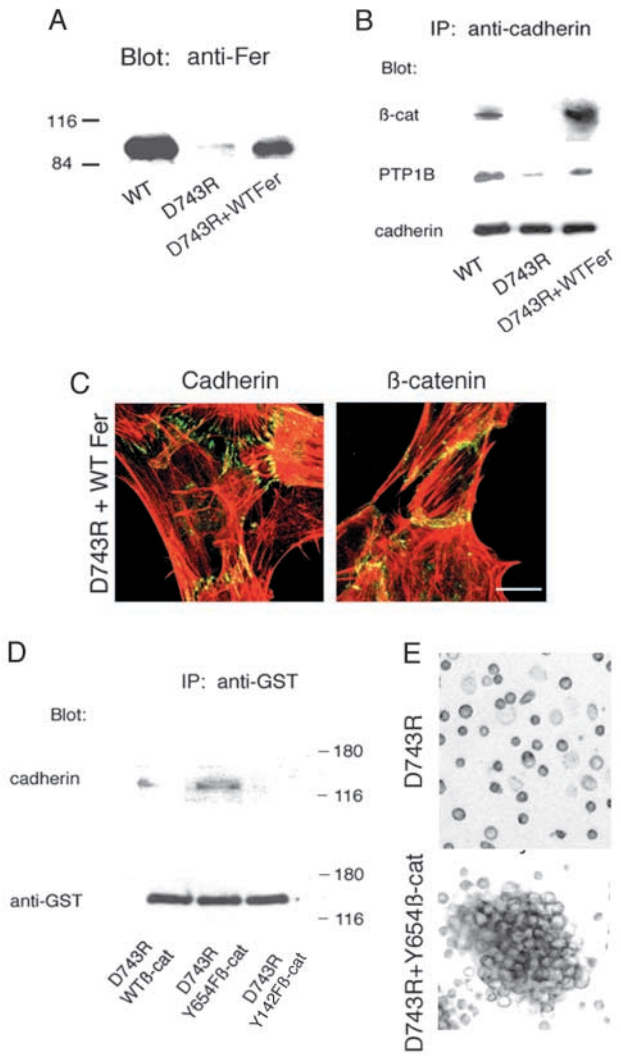

Fig. 9. Rescue of D743R cells by expression of wild-type Fer or Y654F β-catenin. (A) D743R cells were transfected with wild-type Fer cDNA and stable cell lines were selected. Equivalent amounts of protein from cell lysates were analysed by immunoblotting with anti-Fer antibody. 'D743R+WTFer' indicates lysates of D743R cells transfected with wild-type Fer cDNA. (B) WT, D743R and D743R cells transfected with wild-type Fer cDNA (D743R + WT) were lysed in neutral detergent, immunoprecipitated with anti-pan-cadherin antibody and analysed by western blot with anti-β-catenin and anti-PTP1B antibodies. (C) Cells grown on multiple-well slides were fixed, permeabilized and stained with Alexa-568/phalloidin and anti-cadherin or anti-β-catenin antibody followed by Alexa-488-labeled secondary antibody. Notice that cadherin and β-catenin are again localized at cell boundaries in D743R cells expressing wild-type Fer. Scale bar, ~10 μm. (D) D743R cells were incubated with GST/Y654F-β-catenin, GST/wild-type-β-catenin, or GST/Y142F-β-catenin in the presence of BioPorter, the cells were lysed, immunoprecipitated with anti-GST antibody and immunoblotted with anti-pan-cadherin antibody. The immunoblots were stripped and reprobed with anti-GST antibody. (E) D743R cells expressing GST/Y654F-β-catenin were visualized under phase contrast and photographed using an Axiovert 25 microscope and Axiovision (Zeiss) after 3 hours at 37°C in 30 mm dishes rotated at 80 rpm.

dominant-negative inactive form (Balsamo et al., 1998; Rhee et al., 2001) or by introducing into cells a peptide mimicking the cadherin site to which PTP1B binds (Xu et al., 2002), increases the pool of tyrosine-phosphorylated β-catenin, prevents the formation of adhesions and inhibits cadherin-mediated neurite outgrowth. Thus, PTP1B is ideally positioned to routinely dephosphorylate β-catenin, preventing phosphorylation from affecting the stability of cadherin adhesions.

We have used two cell systems to define the role of Fer in cadherin function: primary embryonic chick neural retina cells expressing functional N-cadherin at the cell surface; and immortalized fibroblasts derived from a Fer catalytic null mouse and its wild-type litter mate (Craig et al., 2001). In order to disrupt the association of Fer with the cadherin complex among embryonic retina cells, we have used cell-permeable peptides mimicking the domains through which Fer and p120ctn interact. Disruption of this interaction and loss of bound Fer results in rapid loss of PTP1B, followed by the loss of β-catenin and finally loss of p120ctn. Loss of β-catenin from the cadherin complex correlates with an increase in the pool of tyrosine-phosphorylated β-catenin. By introducing β-catenin with mutations at either tyrosine 142 or 654 into cells, we show that the Y654F mutation compensates for the loss of Fer. Loss of PTP1B from the cadherin complex occurs concomitantly with the loss of Fer and Fer targets PTP1B to the cadherin complex via phosphorylation at tyrosine 152 on PTP1B. These findings strongly imply that PTP1B dephosphorylates β-catenin at tyrosine 654, the crucial residue in maintaining high-affinity β-catenin/cadherin binding (Piedra et al., 2001). Loss of the core cadherin-associated proteins disrupts the cadherin-actin connection and compromises cadherin-mediated adhesions and neurite outgrowth.

To compare the effects of displacing Fer alone with that of p120ctn and associated Fer, we used a cell permeable peptide designed to mimic the p120ctn binding site on cadherin (Thoreson et al., 2000). This peptide (Cad120P) effects the rapid release of p120ctn and thus Fer as well as PTP1B and β-catenin. Concomitant with the loss of β-catenin, cells lose cadherin-mediated adhesion and hyperphosphorylated β-catenin accumulates. Thus, the immediate effects of both Cad120P and FerP on release of components associated with the cytoplasmic domain of cadherin reflect the specificity of the peptides, but the effects on adhesion and phosphorylation of β-catenin are similar and occur within the same time frame. Furthermore, the loss of cadherin-mediated adhesion following treatment with either cell permeable peptide is not due to internalization, because the amount of cell-surface cadherin remains constant.

The absence of Fer in immortalized fibroblasts from mice homozygous for a *fer*^{D743R} mutation results in loss of cadherin-mediated adhesions, either in cells cultured as monolayers or in suspension, and PTP1B is not associated with cadherin. This appears to be due to either an extremely rapid turnover rate of surface cadherin or an inability to mobilize cadherin to the cell surface. The latter possibility is supported by the requirement for β-catenin to transport cadherin efficiently to the cell surface (Chen et al., 1999; Wahl et al., 2003). In tissues derived directly from animals, the amounts of β-catenin and PTP1B associated with cadherin are reduced by approximately 50%. We suggest that this reduction is due to the fact that Fer is absent and the targeting of PTP1B to the cytoplasmic domain of cadherin is limiting, resulting in a reduction in the amount of β-catenin

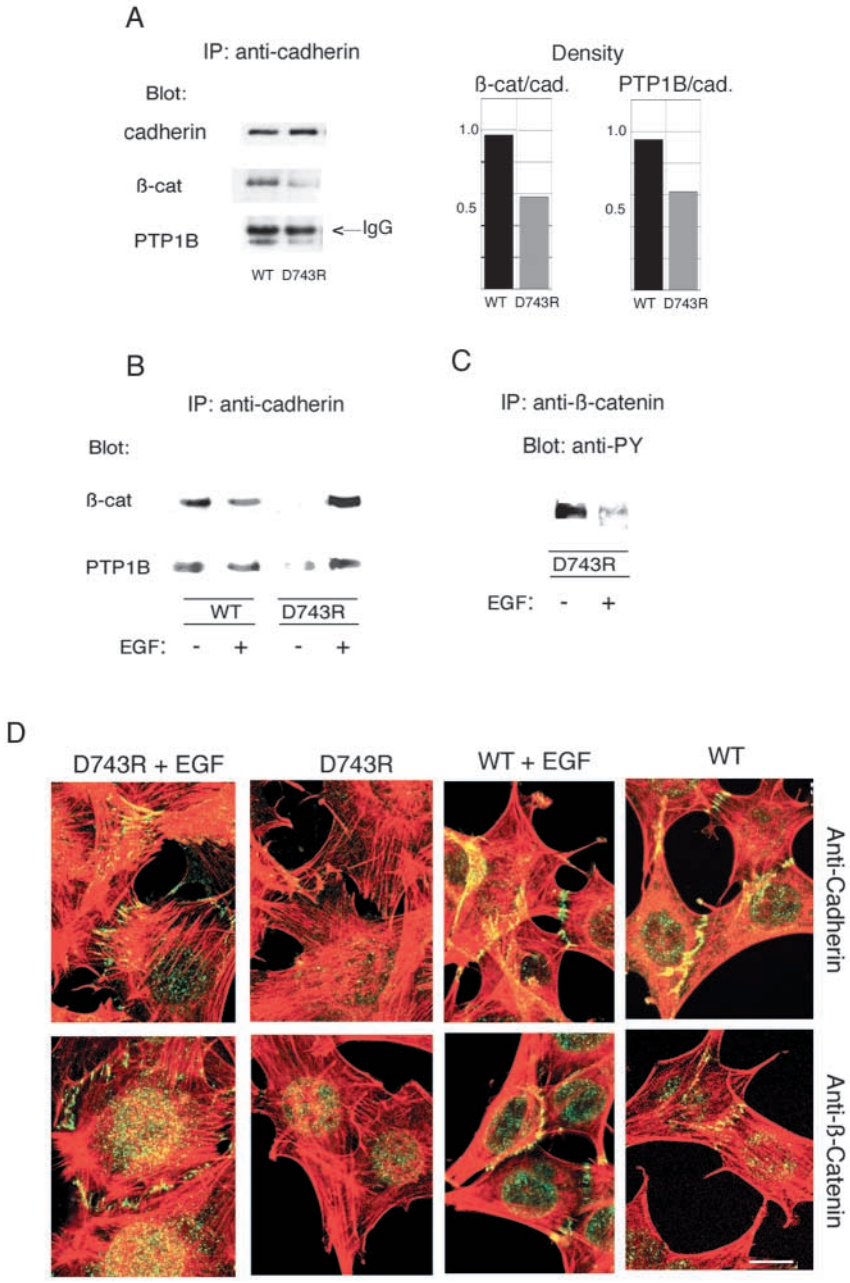

Fig. 10. Association of cadherin with β-catenin and PTP1B in mouse tissue and effect of EGF on D743R cells. (A) Brain lysates from wild-type or *fer*^{D743R} mice were immunoprecipitated with anti-pan-cadherin antibody and analysed by western blot with the indicated antibodies. The relative intensities of the cadherin, β-catenin and PTP1B bands were determined by densitometry and are presented as ratios. (B) WT or D743R cells grown to semiconfluence were washed free of serum and incubated with or without 100 ng ml^{-1} EGF for 2 hours. The cells were then lysed in neutral detergent, immunoprecipitated with anti-pan-cadherin antibody and analysed by western blot with anti-β-catenin and anti-PTP1B antibodies. (C) Alternatively, cells were lysed in RIPA buffer, immunoprecipitated with anti-β-catenin antibody and analysed by western blot with anti-phosphotyrosine antibody.

owing to the accumulation of phosphorylated tyrosine residues. The presence of some PTP1B associated with cadherin in *fer*^{D743R} mouse tissue indicates that there is an in vivo mechanism, which, at least partially, compensates for the lack of functional Fer. Indeed, we have found that, by culturing fibroblasts from *fer*^{D743R} mice in the presence of EGF, cadherin-mediated adhesions with associated PTP1B are restored. Thus, EGF promotes the phosphorylation of PTP1B on tyrosine 152 either directly (Liu and Chernoff, 1997) or indirectly through the activation of another kinase. We suggest that the *fer*^{D743R} mice are able to survive and reproduce owing to the presence of high levels of growth factors during development.

The role of phosphorylation of β-catenin on tyrosine residues as a key regulatory step in the formation and maintenance of the cadherin cytoskeletal connection has at least two dimensions. In addition to the role of Fer in regulating the targeting of PTP1B to cadherin, and thus the steady-state levels of phosphorylated tyrosine residues at position 654, activated Fer can directly phosphorylate β-catenin on tyrosine 142, reducing its affinity for α-catenin (Piedra et al., 2003), and potentially disrupting cadherin-mediated adhesion (Ozawa and Kemler, 1998). Activation of Fer in this case is mediated by the Src family kinase Yes. Thus, Fer appears to play opposing roles in cadherin function: it normally maintains the stability of the cadherin-cytoskeleton link through phosphorylation of PTP1B but, when activated, it decreases the stability of this linkage by directly phosphorylating β-catenin on tyrosine 142. Because Fer is associated with the cadherin complex through p120ctn, these two opposing roles might help to explain some of the differences reported in the role of p120ctn in cadherin function. Among cells expressing enhanced levels or activities of Src-related family members, Fer is activated, its targeting to cadherin is enhanced by the simultaneous phosphorylation of p120ctn (Piedra et al., 2003), and tyrosine 142 on β-catenin is phosphorylated, reducing cadherin-mediated adhesion. Thus, among v-Src-transfected L cells (Ozawa and Ohkubo, 2000) or Colo 205 cells (Aono et al., 1999), which express several activated Src family kinases (Park et al., 1993), compromising the presence or recruitment of activated Fer to the cadherin complex with mutant p120ctn enhances adhesion. Among cells expressing normal levels of src family members, uncoupling p120ctn from cadherin destabilizes adhesions (Thoreson et al., 2000; Ireton et al., 2002). One aspect of this might be a rapid loss of cadherin-mediated adhesion owing to an accumulation of phosphate on tyrosine 654 of β-catenin followed by a slower loss of cadherin itself through internalization and degradation (Davis et al., 2003; Xiao et al., 2003; Huber et al., 2001).

It is interesting that p120ctn, and thus Fer, are not absolutely essential to cadherin-mediated adhesion among vertebrate cell lines, because enhanced expression of cadherin itself can

compensate for loss of p120ctn (Ireton et al., 2002). This might be due to the fact that cadherin is mobilized to the cell surface associated with β-catenin (Chen et al., 1999; Wahl et al., 2003); thus, given a large enough supply of de novo synthesized cadherin/β-catenin complexes, adhesions are maintained. p120ctn is also not essential in the developing *Drosophila* embryo (Myster et al., 2003; Pacquelet et al., 2003). However, null mutations in *Drosophila* p120ctn enhance mutations in DE-cadherin and Armadillo/β-catenin (Myster et al., 2003), suggesting an important role in regulating cadherin-mediated adhesion. One aspect of this regulatory role might well be to act as an adaptor for the DFer tyrosine kinase (Paulson et al., 1997).

In conclusion, cadherin-mediated adhesions are maintained by insuring that β-catenin is continuously dephosphorylated. This requires the presence of tyrosine phosphatase activity (Lilien et al., 2002). In the cells used in these studies, β-catenin is maintained in a dephosphorylated state through the association of both PTP1B and Fer with the cytoplasmic domain of cadherin. Disruption of this balance has profound ramifications for both normal and abnormal development. When overwhelmed by elevated tyrosine kinase activity, β-catenin is tyrosine phosphorylated, cadherin-mediated adhesions are compromised and metastatic potential is increased; this might well be the case among many tumor cells bearing activated forms of Src and its relatives or a misregulated EGFR (reviewed in Mareel and Leroy, 2003). However, under normal conditions, the balance might be altered temporally or spatially to effect important developmental events, such as epithelium-mesenchyme transformations (reviewed in Lilien et al., 2002) and axonal guidance. Development of the precise pattern of axonal trajectories requires many diverse guidance cues (reviewed in Yu and Bargmann, 2001; Grunwald and Klein, 2002). At least two of these cues, the chondroitin sulfate proteoglycan Neurocan (Li et al., 2000) and the glycoprotein Slit (Rhee et al., 2002), result in direct inactivation of N-cadherin by promoting the retention of phosphate or increased phosphorylation of tyrosine residues on β-catenin and hence dissolution of the cadherin-actin linkage. Localized disruption of the cadherin-actin connection is a rapid means of growth-cone response to extracellular cues, collapsing some filopodia while leaving others intact to continue navigation of the growth cone. Thus, although this is only one of several means of regulating cadherin function, its importance might reside in the potential to respond rapidly to environmental cues, be rapidly reversible and result in selective inactivation of only a small, localized population of adhesions within a single cell.

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