p120, a p120-Related Protein (p100), and the Cadherin/Catenin Complex

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Abstract. Cadherins and catenins play an important role in cell–cell adhesion. Two of the catenins, β and γ, are members of a group of proteins that contains a repeating amino acid motif originally described for the *Drosophila* segment polarity gene *armadillo*. Another member of this group is a 120-kD protein termed p120, originally identified as a substrate of the tyrosine kinase pp60^{src}. In this paper, we show that endothelial and epithelial cells express p120 and p100, a 100-kD, p120-related protein. Peptide sequencing of p100 establishes it as highly related to p120. p120 and p100 both appear associated with the cadherin/catenin complex, but independent p120/catenin and p100/catenin complexes can be isolated. This association is shown by coimmunoprecipitation of cadherins and catenins with an anti–p120/ p100 antibody, and of p120/p100 with cadherin or catenin antibodies. Immunocytochemical analysis with a p120-specific antibody reveals junctional colocalization of p120 and β-catenin in epithelial cells. Catenins and p120/p100 also colocalize in endothelial and epithelial cells in culture and in tissue sections. The cellular content of p120/p100 and β-catenin is similar in MDCK cells, but only ~20% of the p120/p100 pool associates with the cadherin/catenin complex. Our data provide further evidence for interactions among the different arm proteins and suggest that p120/p100 may participate in regulating the function of cadherins and, thereby, other processes influenced by cell–cell adhesion.

C ELL adhesion is important for a wide variety of regulatory and developmental processes. The cadherins comprise a family of transmembrane, cell surface glycoproteins that mediate Ca^{2+}–dependent cell–cell adhesion in a homotypic manner (Takeichi, 1991). In cells with well-developed intercellular junctions, the cadherins are localized to the adherens junction (Boller et al., 1985) but appear to influence other intercellular junctions such as gap junctions (Matsuzaki et al., 1990; Musil et al., 1990) and tight junctions (Gumbiner and Simons, 1986; Gumbiner et al., 1988). The adherens junction also plays a crucial role in developing and maintaining cell polarity (see Nelson, 1992), and its dysfunction has been strongly implicated in the invasiveness and carcinogenesis of tumor cells (see, e.g., Behrens et al., 1989; Frixen et al., 1991; Vleminckx et al., 1991; Shimoyama et al., 1992; Hedrick et al., 1993; Tsukita et al., 1993; Birchmeier and Behrens, 1994).

The conserved cytoplasmic domain of cadherins is known to associate with three proteins, termed α-, β-, and γ-catenin (Ozawa et al., 1989), which serve to link cadherins to the actin-based cortical cytoskeleton (Hirano et al., 1987). The association of cadherins with catenins is essential for intercellular Ca^{2+}–dependent adhesiveness (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990; Kintner, 1992). α-catenin

is homologous to vinculin (Herrenknecht et al., 1991; Nagafuchi et al., 1991), making it a good candidate for interaction with the actin-based cytoskeleton (see Ozawa et al., 1990; Hirano et al., 1992). β-Catenin is homologous to the *Drosophila* segment polarity gene *armadillo*, suggesting a role in developmental signaling in vertebrates (McCrea et al., 1991). γ-Catenin is probably identical to plakoglobin (Knudsen and Wheelock, 1992; but see Piepenhagen and Nelson, 1993), which again is homologous to *armadillo* (see Franke et al., 1989; Peifer and Wieschaus, 1990). Indeed, β-catenin and plakoglobin appear to be part of a multigene family (Peifer et al., 1992).

A repeating 42–amino acid motif originally identified in *armadillo* (Riggleman et al., 1989) has also been found in several other proteins, including β-catenin and plakoglobin, with a variety of functions (Peifer et al., 1994). These include the APC gene product, a tumor suppressor protein (Kinzler et al., 1991); p120, a pp60^{src} substrate (Reynolds et al., 1992); smgGDS, an exchange factor for ras-related G proteins (Kikuchi et al., 1992); a suppressor of RNA polymerase I mutations in yeast (Yano et al., 1992, 1994); and band-6 protein, a major desmosomal constituent (Hatzfeld et al., 1994). The function of the repeats in these arm proteins is unknown. Interestingly, the APC gene product associates with β-catenin (Rubinfeld et al., 1993; Su et al., 1993), supporting an important role for catenins in intracellular processes that regulate cell growth. Furthermore, these studies illustrate that cadherins are not exclusive cellular partners of catenins, raising the possibility of other

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interactions among catenins, cadherins, and arm proteins, important in a variety of biological processes.

p120 was initially identified as one of several substrates of the tyrosine kinase $pp60^{src}$ (Reynolds et al., 1989; Kanner et al., 1990). It is membrane associated and can be myristoylated, but does not appear to be glycosylated (Kanner et al., 1991). Mutational analysis suggested that tyrosine phosphorylation of p120 is necessary for $pp60^{src}$-mediated cellular transformation (Linder and Burr, 1988; Reynolds et al., 1989). Although tyrosine phosphorylation of p120 has also been observed in response to epidermal growth factor, platelet-derived growth factor, and colony-stimulating factor 1, and in polyoma virus middle T antigen–transformed cells (Downing and Reynolds, 1991; Kanner et al., 1991), the exact role of p120 in cellular physiology and pathology remains to be established.

For the present report, we studied p120 in endothelial and epithelial cells. First, we provide evidence for the existence of a p120-related 100-kD protein, which we term p100. We then examine the possibility of interaction between p120/p100 and other proteins. Our biochemical data suggest that p120/p100 associates with the cadherin/catenin complexes of endothelial and epithelial cells. Immunocytochemical analysis supports this conclusion. Because p120 appears to be an important $pp60^{src}$ substrate involved in cellular transformation, our data provide a link between tyrosine kinase substrates, cell adhesion molecules, and growth control. p120 and p100 may also be important in modulating of other processes regulated by cell–cell adhesiveness. For example, we recently presented evidence that increased tight-junction permeability was associated with tyrosine phosphorylation of proteins associated with intercellular junctions (Staddon et al., 1995). Clearly, the characterization of kinase substrates at intercellular junctions has implications for a variety of processes influenced by cell–cell adhesion. While the present study was in progress, Reynolds et al. (1994) published similar findings. Our work confirms and extends the observations of Reynolds et al. (1994), linking p120 with the cadherin/catenin complex.

Materials and Methods

Antibodies

The anti–canine E-cadherin antibody rr1, developed by Gumbiner and Simons (1986), was provided by Barry Gumbiner (Memorial Sloan-Kettering Cancer Center, NY) or obtained from the Developmental Studies Hybridoma Bank (maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA) under contract No. N01-HD-2-3144 from the National Institute of Child Health and Human Development (NICHD). The anti–human E-cadherin antibody HECD-1 (Shimoyama et al., 1989) came from Takara Biomedicals (Shiga, Japan). Anti-p120 and anti–focal adhesion kinase (FAK)[1] antibodies came from Transduction Laboratories (Lexington, KY). The anti-p120 antibody 2B12 (Kanner et al., 1990) was a gift from J. T. Parsons (University of Virginia, Charlottesville, VA). The peptide-directed antibodies against α- and β-catenin (Staddon et al., 1995) were kindly provided by Kurt Herrenknecht (Eisai Research Laboratories Ltd., University College London, London, UK). The anti–collagen IV antibody came from Biogenesis (Bournemouth, UK). All secondary antibodies used for immunoprecipitation and immunocytochemistry came from Jackson Laboratories Inc. (West Grove, PA). HRP-conjugated secondary antibodies used for immunoblotting came from Amersham (Buckinghamshire, UK).

Cells

The following cells were cultured at 37°C in a medium containing 100 U/ml penicillin and 100 μg/ml streptomycin: Caco-2 (epithelial cells derived from a human colonic tumor: 5% CO_2, MEM, 10% FCS, 1% nonessential amino acids, 1 μg/ml insulin); CMT 93/69 (epithelial cells derived from a mouse rectal carcinoma: 10% CO_2, DME, 10% FCS); ECV304 (a cell line derived from human umbilical vein endothelial cells: 5% CO_2, M199, 10% FCS); LLC-PK1 (epithelial cells derived from porcine kidney: 5% CO_2, M199, 10% FCS); MDBK (epithelial cells derived from bovine kidney: 5% CO_2, MEM, 10% FCS); Strain I MDCK cells (epithelial cells derived from canine kidney: 5% CO_2, MEM, 10% FCS); RBE4 cells (immortalized rat brain endothelial cells [see Durieu-Trautmann et al., 1993]: 5% CO_2, α-MEM: Ham's F10 [1:1], 10% FCS, 0.3 mg/ml geneticin, 1 ng/ml bFGF); Swiss 3T3 fibroblasts (10% CO_2, DME, 10% FCS). Caco-2, CMT 93/69, ECV304, LLC-PK1 and MDBK cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). MDCK cells were provided by Barry Gumbiner. RBE4 cells came from Pierre Couraud (Université Paris, Paris, France) and Swiss 3T3 fibroblasts came from Enrique Rozengurt (Imperial Cancer Research Fund, London, UK). Human umbilical vein endothelial cells came from Clonetics (Palo Alto, CA) and were cultured according to the manufacturer's instructions. Primary cultures of bovine and porcine brain endothelial cells were grown as described by Rubin et al. (1991). For experimental purposes, confluent cultures of Caco-2, MDBK, MDCK, and brain endothelial cells were established on tissue culture–treated, polycarbonate Transwell filters (polycarbonate, 0.4 μm; Costar, Cambridge, MA). Other cells were grown on tissue culture plastic.

Immunoblotting and Immunoprecipitation

Whole-cell lysates from cultures maintained 16–20 h in 0.5% serum were prepared by rapidly replacing the medium with hot Laemmli sample buffer (Laemmli, 1970) supplemented with 5 mM EDTA, followed by heating at 100°C for 5 min. Proteins were resolved by slab-gel electrophoresis as described by Laemmli (1970). The gels were equilibrated in buffer containing 48 mM Tris, 39 mM glycine, 0.03% SDS (wt/vol) and 20% methanol (vol/vol), and then transferred to nitrocellulose filters (Hybond ECL; Amersham). After Ponceau S staining, the filters were blocked in 5% (wt/vol) nonfat dried milk in PBS at 4°C for 16–18 h. Filters were then incubated for 1 h with primary antibody in PBS containing 0.05% Tween 20 and 1% BSA, followed by detection with appropriate HRP-conjugated secondary antibody and chemiluminescence (ECL; Amersham).

Immunoprecipitations were performed at 4°C. Cultures were rinsed with PBS and then lysed in either TX buffer (1% (vol/vol) Triton X-100, 25 mM Hepes, 2 mM EDTA, 0.1 M NaCl, 25 mM NaF, 1 mM vanadate, 25 μM phenylarsine oxide, pH 7.6 [adjusted with NaOH], 1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 0.1 U/ml α2-macroglobulin, 10 μg/ml leupeptin) or TDS buffer, which was identical to the TX buffer except that it was supplemented with 0.5% (wt/vol) sodium deoxycholate and 0.2% (wt/vol) SDS. The cells were incubated with lysis buffer for 10–15 min and then scraped. The lysates were collected and centrifuged at 14,000 g for 20 min. The supernatant was precleared with protein A Sepharose (Pharmacia, UK) for 1–2 h and then incubated with primary antibody for 1 h followed by an additional 1 h with protein A Sepharose alone, in the case of rabbit antibodies, or together with rabbit anti–mouse antibodies for the mouse monoclonal antibodies. After five washes in lysis buffer, immune complexes were dissociated by adding of Laemmli sample buffer, followed by heating at 100°C for 5 min. Protein analysis was performed using SDS-PAGE, and immunoblotting was performed as described earlier.

For the analysis of stoichiometry (see Fig. 6), MDCK cells were lysed in 1 ml TX buffer per 10 cm² of filter. Immunoprecipitations were performed with either 5 μg of the β-catenin antibody or 10 μg of the anti-p120 antibody per ml of lysate. After an initial centrifugation to collect the immune complex, the supernatant, containing protein that was not immunoprecipitated, was collected and mixed with 0.33 volumes of 4× concentrated SDS sample buffer. The immune complex was washed as usual, and proteins were collected in sample buffer that was the same in volume as the collected supernatant. Equal volumes of the immunoprecipitated proteins and the supernatant (equivalent to 0.7 cm² filter area) were analyzed by SDS-PAGE and immunoblotting using anti–β-catenin antibody (0.1 μg/ml) or the anti-p120 antibody (0.25 μg/ml).

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1. *Abbreviation used in this paper:* FAK, focal adhesion kinase.

For [^{35}S]methionine labeling, the cultures were washed twice in methionine-free MEM supplemented with 0.5% FCS. The cells were incubated for 16–18 h in this medium containing 50 μCi/ml [^{35}S]methionine (>1,000 Ci/mmol; Amersham). Protein analysis was performed using SDS-PAGE, followed by fixation in 25% methanol/10% acetic acid. Labeled protein was detected either by direct autoradiography at room temperature or by fluorography at −80°C following impregnation of the gel with Amplify (Amersham). For quantitative detection of proteins by either fluorography or enhanced chemiluminescence, films were preflashed (Laskey and Mills, 1975). Integrated band density was determined by densitometry.

Microsequencing

Confluent cultures (20-×-9-cm dishes) of Caco-2 cells were washed three times with ice-cold PBS. The cells were lysed in TX buffer (1 ml/dish) at 4°C, conditions which minimize nuclear lysis but extract p120 and p100 in association with catenins. After the dishes were scraped, the pooled lysate was centrifuged at 10,000 g for 20 min. Sodium deoxycholate and SDS were added to the supernatant to final concentrations of 0.5% and 0.2%, respectively, thereby dissociating p120 and p100 from catenins. This TDS lysate was precleared with protein A Sepharose (1 ml of a 10% [wt/vol] suspension) for 1 h. Anti-p120 antibody (50 μg) was then added to immunoprecipitate p120 and the immunologically related p100. After 2 h, the immune complex was collected using 100 μg of rabbit anti–mouse IgG and 1 ml of protein A Sepharose. The beads were washed five times with TDS buffer. Immunoprecipitated protein was eluted into 1 ml of Laemmli sample buffer, followed by heating at 100°C for 5 min. Protein was then precipitated using 4 volumes of ethanol followed by 16 h incubation at −20°C. The protein precipitate was collected by centrifugation at 4,000 g for 30 min at 4°C and then solubilized in 50 μl of 2× concentrated Laemmli sample buffer followed by heating at 100°C for 5 min. Protein was resolved on a 0.75-mm thick 6% polyacrylamide gel, which was fixed in 25% (vol/vol) methanol/0.5% (v/v) acetic acid for 5 min, followed by staining with 0.2% (wt/vol) Coomassie brilliant blue for 1 h. The gel was de-stained in 30% methanol. A slice of polyacrylamide containing stained protein corresponding to p100 (see Fig. 1) was excised from the gel and digested in situ with lysylendopeptidase C, according to Ferrara et al. (1993), except that (1) the "dehydration buffer" contained 50 mM Tris-Cl, pH 8.5, 1 mM EDTA, 50% acetonitrile, and the "rehydration buffer" contained 50 mM Tris-Cl, pH 8.5, 1 mM EDTA, 0.02% Tween 20; and (2) digestion with lysylendopeptidase C was allowed to proceed overnight at 37°C, with shaking on an Eppendorf Thermomixer (Eppendorf North America, Madison, WI), with 50 pmole protease added at time zero and after 4 h. Peptide fragments were extracted as described with 0.1% TFA and 60% acetonitrile, and the organic solvent was removed by vacuum centrifugation. The fragments were subsequently captured on a peptide trap cartridge (Michrom BioResources, Inc., Auburn, CA) and then purified on a Reliasil RP-18 column (1 × 150 mm) at 50 μl/min using Michrom UMA HPLC (Michrom BioResources, Inc.). A 60-min linear gradient

from 10% "B" to 70% "B" was employed where solvent A contained 0.1% TFA, 2% acetonitrile, and solvent B was composed of 0.1% TFA, 90% acetonitrile. RPLC-purified digestion fragments were subjected to microsequence analyses on a G1000 protein sequencer (Hewlett-Packard Co., Palo Alto, CA) using version 2.2 chemistry and software.

Immunocytochemistry

For the majority of experiments, cells were fixed at room temperature for 15 min in 3% paraformaldehyde made up in PBS containing 0.5 mM CaCl$_2$ and 0.5 mM MgSO$_4$. Fixed cells were washed and then permeabilized by incubation with 0.5% Triton X-100 in PBS for 10 min. After washing, the cells were incubated for 30 min in PBS containing 10% calf serum and 0.1 M lysine, pH 7.4. Incubation with primary antibody was in PBS containing 10% calf serum for 1 h. After washing, the cells were incubated for 30–60 min with a 1:100 dilution of fluorophore-conjugated anti–mouse or anti–rabbit IgG, as appropriate, in PBS containing 10% calf serum. After washing, the filters were mounted with Citifluor (Citifluor Products, Canterbury, UK) and examined using a Microphot-FXA fluorescence microscope (Nikon Inc., Melville, NY) fitted with 40× and 60× objectives. Photographs were taken using Kodak T-MAX film (400 ASA; Eastman Kodak, Rochester, NY).

Where indicated, unfixed cells were also labeled after permeabilization with digitonin. Cultures were washed three times at 4°C with PBS lacking calcium and magnesium. They were then incubated at 4°C for 5 min in K medium (see Staddon et al., 1990) containing 0.007% (wt/vol) digitonin, washed three times in K medium, and then labeled with antibodies as described earlier. After staining, the cells were fixed in paraformaldehyde for 15 min, washed, and examined.

For preparing cryosections, brain and skeletal muscle from CO$_2$-asphyxiated rats were removed and rapidly frozen in liquid nitrogen. Tissue blocks were mounted in Tissue Tek (R. Lamb, London, UK), and sections of 5–10 μm thickness were cut on a cryostat (Bright Instrument Company Ltd., Cambridge, UK), air dried, and stored for up to 4 wk at −20°C. After thawing, the sections were fixed and permeabilized as described earlier. The sections were then washed, blocked with PBS containing 10% calf serum for 15 min and incubated with primary antibody diluted in PBS containing 10% calf serum for 2 h. After washing, they were incubated with PBS containing 10% calf serum with either 10% goat serum or 10% donkey serum, as appropriate for the host of the secondary antibody, for 15 min. They were then incubated with secondary antibody diluted in PBS and serum for 1 h. Sections were washed, mounted, and examined as described previously.

Results

Antibody Characterization: p120 and p100 Proteins

SDS lysates of a variety of cell types were analyzed by im-
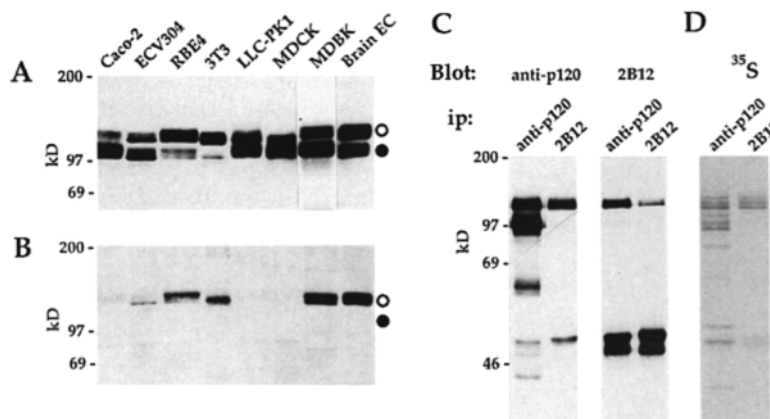
Figure 1. Characterization of the anti-p120 and 2B12 antibodies. (*A* and *B*) Various cell lines and primary cultures of bovine brain endothelial cells (Brain EC) were lysed in SDS sample buffer and separated by SDS-PAGE, followed by immunoblotting with anti-p120 antibody (*A*) or 2B12 antibody (*B*). The exposure times were 1 min for (*A*) (apart from that for the MDBK cells, which was 10 s) and 15 min for (*B*). The migrations of p120 (○) and p100 (●) are indicated. (*C*) p120 protein is recognized by the anti-p120 antibody and cross-reacts with the 2B12 antibody, whereas p100 is recognized only by the anti-p120 antibody. MDBK cells were lysed in TDS buffer. Immunoprecipitations were performed using the anti-p120 antibody or 2B12, followed by cross-blotting. MDBK cells were chosen because p120 and p100 are well separated by SDS-PAGE and react with both antibodies. (*D*) MDBK cells were labeled with [^{35}S]methionine, lysed in TDS buffer, and then immunoprecipitated using anti-p120 or 2B12. Proteins were separated by SDS-PAGE and detected by autoradiography. Clearly, the broad bands (see *A* and *C*) corresponding to p120 and p100 as detected by immunoblotting are resolved as multiple bands. The same bands seen in the 2B12 immunoprecipitate (○) are seen in the anti-p120 immunoprecipitates. In the anti-p120 immunoprecipitates, additional bands (●) corresponding to p100 are also observed.
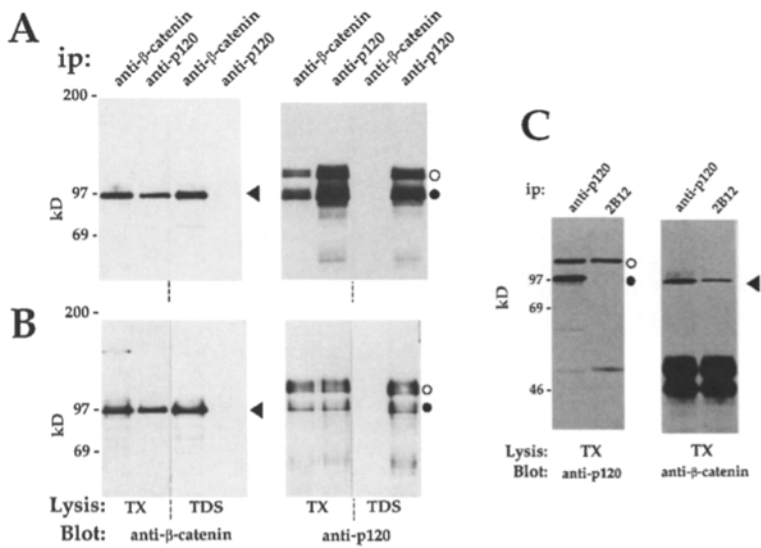
Figure 2. Detection of p120/p100 in β-catenin immunoprecipitates and β-catenin in anti-p120 immunoprecipitates from endothelial cells. ECV304 cells (*A*) and human umbilical vein endothelial cells (*B*) were lysed in either TX or TDS buffer. Lysates were immunoprecipitated using anti–β-catenin or the anti-p120 antibody, followed by analysis using SDS-PAGE and immunoblotting. The migration of β-catenin (*arrowheads*), p100 (●), and p120 (○) have been indicated. Note the absence of anti–p120 reactive material in the β-catenin immunoprecipitates obtained using TDS buffer. Even though β-catenin and p100, as defined by its reactivity with anti-p120 antibody, migrate very closely, they are clearly distinct proteins. (*C*) Distinct β-catenin–p120 and β-catenin–p100 complexes. ECV304 cells were lysed in TX buffer and immunoprecipitated with the anti-p120 antibody, which recognizes p120 and p100, or 2B12, which only recognizes p120. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with the anti-p120 antibody or β-catenin antibody. Clearly, 2B12 immunoprecipitated p120 and coprecipitated β-catenin but not p100. Therefore, p100 does not associate with a p120/β-catenin complex, implying the existence of a separate p100/β-catenin complex.

munoblotting for expression of p120 using an anti-p120 antibody (Fig. 1 *A*). This antibody was raised against a polypeptide containing amino acids 790–911 at the COOH terminal of p120. The antibody clearly reacted with two distinct bands of apparent molecular mass 120 and 100 kD in extracts of Caco-2, ECV 304, RBE4, Swiss 3T3, LLC-PK1, MDBK, and brain endothelial cells. Hereafter, we refer to these two bands as p120 and p100. With MDCK cells, a broad continuous band was detected. The relative intensity of staining of p120 and p100 depended on the cell type. For example, p100 was more intense than p120 in Caco-2 cells, whereas in the Swiss 3T3 cells the reverse was observed. These blots were also probed with the monoclonal anti-p120 antibody 2B12, raised against a phosphotyrosine immunoprecipitate prepared from pp60^{src}–transformed chicken embryo fibroblasts (Kanner et al., 1990). This antibody recognized only the upper of the two bands (Fig. 1 *B*). 2B12 reacted strongly with bovine protein, moderately with rodent protein, and weakly with human protein. However, it failed to react under these conditions with whole-cell extracts of the LLC-PK1 (porcine) and MDBK (canine) cells, suggesting limited cross-species reactivity in immunoblot analysis.

The relationship between the known pp60^{src} substrate p120 and p100 was further examined by immunoblot analysis of immunoprecipitates from cells lysed in TDS buffer, used to minimize protein–protein interaction (Fig. 1 *C*). MDBK cells were used as an example because both the anti-p120 and 2B12 antibodies reacted well with protein from these cells (Fig. 1, *A* and *B*). In the anti-p120 immunoprecipitates, p120 and p100 were detected by blotting with the anti-p120 antibody. 2B12 only reacted with p120 in these immunoprecipitates. In 2B12 immunoprecipitates, blotting with both 2B12 and the anti-p120 antibody revealed p120 (Fig. 1 *C*). Thus, 2B12 recognized p120, whereas the anti-p120 antibody recognized the same p120 protein as did 2B12 and, in addition, a p100 protein.

Autoradiographic analysis of immunoprecipitates of [^{35}S] methionine-labeled MDBK cells, as resolved by SDS-PAGE

(Fig. 1 *D*), revealed that the p120 protein migrated as a cluster of at least three bands that were identical in both the 2B12 immunoprecipitates and the anti-p120 immunoprecipitates. However, in the anti-p120 immunoprecipitates, a similar cluster of additional bands corresponding to p100 was detected. Technically, detection by immunoblotting (chemiluminescence) is light based, and resolution is not as good as when using direct autoradiography; hence, the multiple bands seen in Fig. 1 *D* appear as a broad band in Fig. 1 *C*. The basis of the multiplicity of the bands corresponding to p120 and p100 in the MDBK cells is unclear. [^{32}P]Phosphate-labeling of these cells raised the possibility that these bands may represent differentially phosphorylated protein, because they all labeled with phosphate. However, dephosphorylation of the proteins in the immunoprecipitate with potato acid phosphatase prior to electrophoresis (see Meisenhelder and Hunter, 1991) did not affect their migration (results not shown). Also, the ability to resolve p120 and p100 into multiple bands depended on the cell type from which they were isolated (e.g., see data for MDCK cells, later in section).

It is possible that p120 and p100 share an epitope recognized by the p120 antibody but are otherwise unrelated. To extend the analysis of the relationship between these proteins, we immunoaffinity purified p100 from human epithelial cells and determined the amino acid sequence of peptides derived from this protein. As shown in Table I,

Table I. Peptide Sequences of Human Epithelial p100/Lys C Digestion Fragments

Mouse p120	Mouse p120 sequence	Human p100 sequence
434–444	NISFG RDQDN K	NI(S)FG RDQDN K
744–751	HARPN LV	HAIPN LV
799–804	LVLIN K	XVLIN K

Amino acid sequences (single-letter code) of peptides derived from p100 purified from Caco-2 cells were as described in Materials and Methods. Sequence differences between the mouse p120 (Reynolds et al., 1992) and human p100 sequences are accentuated in bold, underlined type. Tentative sequence assignments are indicated by parentheses. X, unidentified amino acid.

the sequences we obtained are highly homologous with regions of the known mouse p120 protein (Reynolds et al., 1992). These homologous sequences were identified both within and without the immunological epitope defined by the antibody used to immunoprecipitate and purify p100. Additionally, the three sequences we obtained are contained in the third, ninth, and tenth arm repeats of p120, as defined by Peifer et al. (1994). Some but not all of the amino acids of p100 correspond to those of the universal consensus for the arm repeat (see Peifer et al., 1994). To date we have not determined whether the sequence difference we observed between the known mouse p120 sequence and our human p100 sequence is a species–specific difference or whether the two proteins, p120 and p100, are different but highly related. However, p100 is clearly an arm protein.

Association with the Cadherin/Catenin Complex: Endothelial Cells

Given that certain members of the arm protein family are known to interact with other proteins, we next explored the possibility that p120/p100 might associate with other proteins. Endothelial cells form junctional complexes similar in composition to those found in epithelial cells (for review see Rubin, 1992). As shown in Fig. 1, A and B, they also contain p120 and p100 proteins well separated by SDS-PAGE. Using [35S]methionine-labeling, we found that p120 and p100 were the major proteins in the anti-p120 immunoprecipitates from TDS-lysed ECV304 cells and human umbilical vein endothelial cells (results not shown; they were, however, similar to those obtained with epithelial cells; see Fig. 1 D). These proteins are clearly detected by immunoblotting in such immunoprecipitates, but β-catenin was absent (Fig. 2, A and B). Conversely, β-catenin was observed in anti–β-catenin immunoprecipitates from TDS lysates, but p120 and p100 were absent. However, immunoprecipitation of lysates prepared in the milder TX buffer revealed that p120/p100 could associate with other proteins. Thus, in the β-catenin immunoprecipitates, β-catenin together with p120 and p100 were detected. In the anti-p120 immunoprecipitates, p120 and p100 were detected together with β-catenin (Fig. 2, A and B). Thus, anti–β-catenin antibody coimmunoprecipitates p120 and p100, and anti-p120 antibody coimmunoprecipitates β-catenin from TX-, but not TDS-, lysed cells.

Although β-catenin and p100 seen in the immunoprecipitates have a similar mobility (Fig. 2, A and B), this does not represent cross-reactivity of the anti-p120 antibody with β-catenin. Clearly, the anti-p120 antibody does not react with β-catenin in β-catenin immunoprecipitates from TDS-lysed cells. Conversely, β-catenin antibody does not react with p100 in anti-p120 immunoprecipitates from TDS-lysed cells. Therefore, it is unlikely that the β-catenin antibody coimmunoprecipitates p120 and p100 by cross-reacting with these proteins and vice versa.

By [35S]methionine-labeling, in both anti-p120 and anti–β-catenin immunoprecipitates from [35S]methionine-labeled, TX-lysed ECV304 cells, human umbilical vein endothelial cells and brain endothelial cells, bands corresponding to α- and β-catenin and higher molecular weight bands (~130–140 kD) perhaps corresponding to cadherins (see,

e.g., Liaw et al., 1990; Lampugnani et al., 1992; Salomon et al., 1992) were detected (results not shown).

These experiments, although clearly indicating an interaction between p120/p100 and catenins, could not distin-
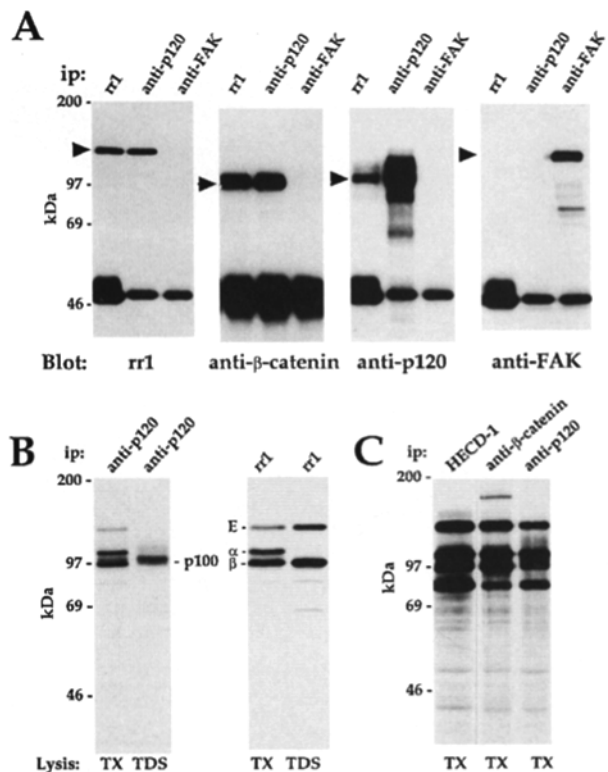
Figure 3. Detection of anti-p120 reactive material in anti–E-cadherin immunoprecipitates and E-cadherin and β-catenin in the anti-p120 immunoprecipitates from epithelial cells. (*A*) MDCK cells were lysed in TX buffer. Immunoprecipitations were performed with either antibody to E-cadherin (rr1), the anti-p120 antibody, or anti-FAK. Following separation by SDS-PAGE, parallel blots were probed using rr1 (*arrowhead*; E-cadherin), anti–β-catenin (*arrowhead*; β-catenin), anti-p120 (*arrowhead*; anti-p120 reactivity), and anti-FAK (*arrowhead*; FAK). Clearly, rr1 and the anti-p120 antibody immunoprecipitated E-cadherin and β-catenin. rr1 could also immunoprecipitate anti-p120 immunoreactive material, but to a lesser extent than that immunoprecipitated by the anti-p120 antibody. FAK could only be immunoprecipitated by anti-FAK. (*B*) and (*C*) Comparison of anti-p120 and anti–E-cadherin immunoprecipitates from [35S]methionine-labeled MDCK and Caco-2 cells. MDCK cells (*B*) were lysed in either TX buffer or TDS buffer. Immunoprecipitations were performed using anti-p120 or anti–E-cadherin (rr1). Proteins were separated by SDS-PAGE followed by fluorography. Bands corresponding to E-cadherin (E), α-catenin (α), and β-catenin (β), which are seen strongly in the rr1 immunoprecipitates, have been indicated. The major anti-p120 reactive band migrates at approximately the position of that of p100 (see Fig. 1 *A*). 2B12 does not appear to react well in immunoblots with canine protein (see Fig. 1 *B*), making positive identification of p120 difficult. Caco-2 cells (*C*) were lysed in TX buffer, and immunoprecipitations were performed using anti–E-cadherin (HECD-1), anti–β-catenin, or anti-p120 antibodies. Proteins were separated by SDS-PAGE followed by fluorography. In all cases, four major bands were immunoprecipitated, corresponding in order of increasing mobility to E-cadherin and α-, β-, and γ-catenin (on the basis of immunoblotting; results not shown).

guish between the possible existence of a catenin/p120/p100 complex or independent catenin/p120 and catenin/p100 complexes. We therefore compared immunoprecipitates from TX-lysed ECV304 cells using either the anti-p120 antibody or the 2B12 antibody. Both antibodies coimmunoprecipitated β-catenin (Fig. 2 C). However, the anti-p120 antibody immunoprecipitated p120 and p100, as expected, but 2B12 immunoprecipitated only p120. The absence of p100 in the 2B12 immunoprecipitate implies that independent p120/catenin and p100/catenin complexes exist, because 2B12 should have immunoprecipitated p100 if a catenin/p120/p100 complex was present.

Association with the Cadherin/Catenin Complex: Epithelial Cells

We next explored the possibility of association of p120/p100 with the cadherin/catenin complex in epithelial cells. MDCK cells were lysed in TX buffer and immunoprecipitated with the anti–E-cadherin antibody rr1, anti–p120 antibody, or, as a negative control, anti–focal adhesion kinase (anti-FAK). The identity of proteins in the E-cadherin and anti-p120 immunoprecipitates was examined by immunoblotting (Fig. 3 A). As expected, E-cadherin and β-catenin were both present in the rr1 immunoprecipitates. An anti-p120 reactive band displaying a mobility similar to that of β-catenin was also present in the rr1 immunoprecipitates. In the anti-p120 immunoprecipitates, E-cadherin and β-catenin were clearly detected as well as a relatively greater amount of anti-p120 reactive material than seen in the rr1 immunoprecipitates. As a negative control, immunoprecipitation with anti-FAK antibody, of the same species and isotype as the anti–p120 antibody, immunoprecipitated FAK but not E-cadherin, β-catenin, or anti-p120 reactivity (Fig. 3 A). Therefore, it appears that, under the conditions of the experiment, anti-p120 immunoreactive material, apparently mainly p100, is associated with the E-cadherin complex. However, in the anti-p120 immunoprecipitates, more p120/p100 is immunoprecipitated than in the rr1 immunoprecipitates, indicating that a pool of p120/p100 exists that is not associated with the cadherin/catenin complex.

When [35S]methionine labeling was used, the major band seen in anti-p120 immunoprecipitates from TDS lysates of MDCK cells corresponded to the broad band detected by immunoblotting (cf. Figs. 1 A and 3 B). In contrast, the anti-p120 immunoprecipitates from MDCK cells lysed in TX buffer, used to preserve macromolecular protein complexes, revealed additional distinct bands at ~130, 105, and 97 kD (Fig. 3 B). Proteins of similar molecular mass were seen in E-cadherin immunoprecipitates from similarly lysed cells (Fig. 3 B). In both cases, as confirmed by immunoblotting, the 130-kD band corresponded to E-cadherin, the 97-kD band to β-catenin, and the 105-kD band to α-catenin, which is dissociated from the complex by lysis in TDS buffer (see McCrea and Gumbiner, 1991).

The p120 antibody does not cross-react with α- or β-catenin or E-cadherin in these cells. Thus, lysis in TDS buffer results in dissociation of the E-cadherin/catenin/p120/p100 complex into an E-cadherin/β-catenin complex, free α-catenin (see McCrea and Gumbiner, 1991) and free p100/p120. Under these conditions, rr1, anti–α-catenin,

anti–β-catenin, and anti-p120 antibodies only immunoprecipitate their primary antigens (except for the coimmunoprecipitation of β-catenin with anti–E-cadherin, and vice versa). Cross-reactivity with γ-catenin, a protein of ~85 kD, is unlikely, because the immunoblots in Fig. 1 fail to show reactivity with protein below 100 kD. We have also observed the anti-p120 immunoreactive band, as seen in the rr1 immunoprecipitates (Fig. 3 A), in α-catenin (results not shown), and in β-catenin immunoprecipitates (see later section).

To explore the generality of the observations made with MDCK cells, Caco-2 cells were also [35S]methionine labeled and lysed in TX buffer, and immunoprecipitations were performed using another anti–E-cadherin antibody HECD-1, anti–β-catenin antibody, or the anti-p120 antibody. HECD-1 or anti–β-catenin clearly immunoprecipitated four major bands corresponding in order of increasing mobility to E-cadherin, α-, β-, and γ-catenin. Similar bands were immunoprecipitated, but to a lesser extent, with the anti-p120 antibody (Fig. 3 C). These results (not shown) were also obtained in MDBK cells when β-catenin immunoprecipitates were compared with those obtained with the anti-p120 antibody. Thus, it appears that the anti-p120 antibody can immunoprecipitate proteins that comigrate with those of the cadherin/catenin complex from a variety of epithelial cell lines as well as from different types of endothelial cells.

With respect to the detection of catenins and p120/p100 by [35S]methionine labeling and fluorography in the MDCK cells, the catenins are easier to detect because their methionine content is approximately twice that of p120 (and we assume that p100 is similar in methionine content to p120). Furthermore, the catenins migrate as discrete bands
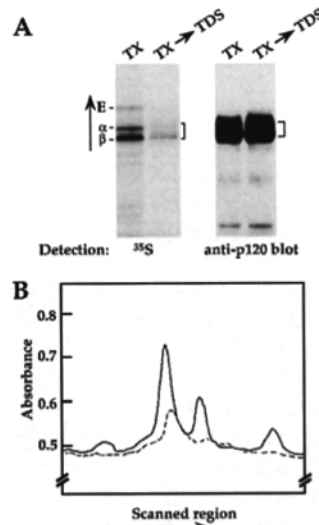
Figure 4. The [35S]methionine labeling of catenins dominates that of p120/p100. MDCK cells were [35S]methionine labeled for 16 h, lysed in TX buffer, and immunoprecipitated in duplicate with the anti-p120 antibody. SDS sample buffer was added to one of the immunoprecipitates (TX). The other (TX→TDS) was mixed with TDS buffer for 1 h, followed by washing in TDS buffer and then extraction into SDS sample buffer. In this manner, p120/p100 was retained by the antibody in the immune complex, whereas the catenins and E-cadherin were dissociated. The samples were analyzed by SDS-PAGE, followed by detection of proteins by fluorography (A, left) or immunoblotting with the anti-p120 antibody (A, right). The migration of p120/p100 is indicated by the bracket. Labeled bands corresponding to E-cadherin (E), α-catenin (α), and β-catenin (β) are indicated. The cadherin/catenin/p120/p100 region of the gel was scanned by densitometry (B). The scans (in register) show the TX lane (continuous line) and TX→TDS lane (dashed line).
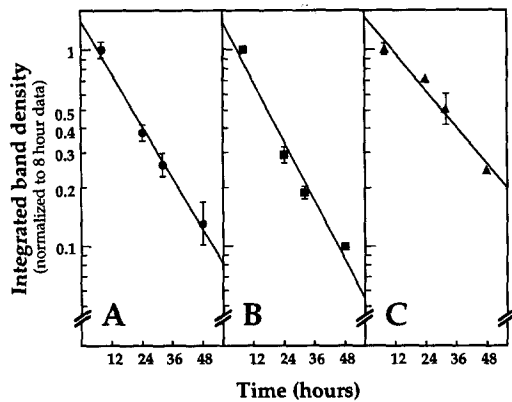
Figure 5. Metabolic half-lives of β-catenin, E-cadherin, and p120/p100. MDCK cells were labeled for 16 h with [^{35}S]methionine, chased with unlabeled methionine, and then lysed at the indicated times in TX buffer. After centrifugation and preclearance with Protein A Sepharose, SDS and deoxycholate were added to final concentrations of 0.2% and 0.5%, respectively. β-catenin was immunoprecipitated using the anti–β-catenin antibody, and p120/p100 were immunoprecipitated using the anti-p120 antibody. E-cadherin was coimmunoprecipitated with β-catenin. Under these detergent conditions, p120/p100 is dissociated from the cadherin/catenin complex. Following SDS-PAGE and fluorography using flashed film, the integrated density of the bands corresponding to β-catenin, E-cadherin, and p120/p100 (as seen, for example, in Fig. 3) were determined by densitometry. The values shown are the means ± SD of immunoprecipitations from triplicate filters of cells. Where the error bars are not apparent, they were too small to clearly depict. The half-lives are β-catenin, 13.7 h (*A*); E-cadherin, 12.0 h (*B*); and p120/p100, 20.0 h.

in SDS–polyacrylamide gels. In MDCK cells, p100 is expressed to a greater extent than p120 and migrates as a diffuse band, further reducing the intensity of exposure of the fluorogram. The results depicted in Fig. 4 illustrate the problem of detection. MDCK cells were labeled with [^{35}S]methionine, lysed in TX buffer, and immunoprecipitated with the anti-p120 antibody. The immunoprecipitates were analyzed by SDS-PAGE, followed by fluorography (Fig. 4 *A*, *left*) and anti-p120 blotting (Fig. 4 *A*, *right*). Another identical anti-p120 immunoprecipitate prepared from the same lysate was mixed with TDS buffer for 1 h and then washed. The proteins remaining in the immune complex were also analyzed. In this manner, p120/p100 was retained in the immune complex, and catenins and cadherins were dissociated. The major bands seen in the fluorogram from the TX immunoprecipitate correspond to E-cadherin, α-catenin, and β-catenin, but it is difficult to discern any labeling due to p120/p100 (Fig. 4). However, removing greater than 95% of the catenins from the anti-p120 immune complex (verified by immunoblotting; results not shown) reveals labeling attributable to p120/p100. The anti-p120 immunoblots indicate that p120/p100 in both the anti-p120 immune complex and the catenin-depleted anti-p120 immune complex are very similar. Densitometry of the fluorogram (Fig. 4 *B*) demonstrates quantitatively the relationship between band intensities of the catenins and p120/p100. Clearly, the labeling due to β-catenin dominates that of p100.

Stoichiometry of the Cadherin/Catenin/p120/p100 Complex

The data presented so far do not indicate how much p120/p100 is in the cadherin/catenin complex in relation to the cadherins and catenins. We assessed the stoichiometric relationship between β-catenin and p120/p100 in the cadherin/catenin complex in MDCK cells. In these cells, p100 was expressed to a much greater extent than p120. Furthermore, because the two proteins were not well resolved electrophoretically, data with respect to p120/p100 were combined for this analysis. First, the relative amounts of β-catenin and p120/p100 were determined by [^{35}S]methionine labeling, correcting for differences in metabolic half-lives, efficiencies of immunoprecipitation, and methionine content. Cells were labeled with [^{35}S]methionine for 16 h, extracted into TX buffer, and immunoprecipitated under TDS buffer conditions with the anti–β-catenin antibody or the anti-p120 antibody. Following SDS-PAGE
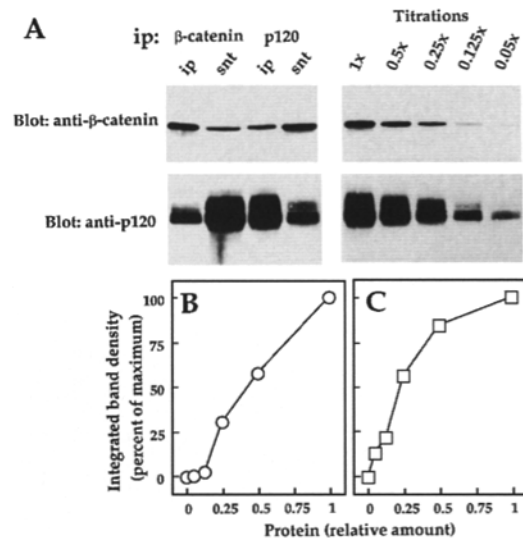
Figure 6. Analysis of stoichiometry of p120/p100 in the cadherin/catenin complex. (*A, left*) MDCK cells were lysed in TX buffer and immunoprecipitated with either the β-catenin antibody or the anti-p120 antibody. Protein in the immunoprecipitate (ip) and that remaining in the supernatant (snt) were analyzed by immunoblotting. (*A, right*) Decreasing known amounts of the protein in the immunoprecipitate—β-catenin from the β-catenin immunoprecipitate (*upper*) and p120/p100 from the anti-p120 immunoprecipitate (*lower*)—allowed the determination of the amount of protein in the immunoprecipitate relative to that remaining in the supernatant. By measuring the integrated density of the bands shown in (*A, left*), the amount of protein in the immunoprecipitate relative to that in the supernatant could be determined by referring to *B* (β-catenin) and *C* (p120/p100), which show the relative integrated density of the bands in *A* (*right*). In this way, no assumptions are made with respect to band intensity and amount of protein. The data shown in this figure are typical of those obtained with the usual conditions employed in the present study. By increasing the ratio of antibody to the number of extracted cells, 100% efficient immunoprecipitation could be achieved. In this case, the stoichiometric relationship with respect to the amount of p120/p100 in a β-catenin immunoprecipitate, and vice versa, was very similar to that obtained from the data presented in this figure (results not shown).
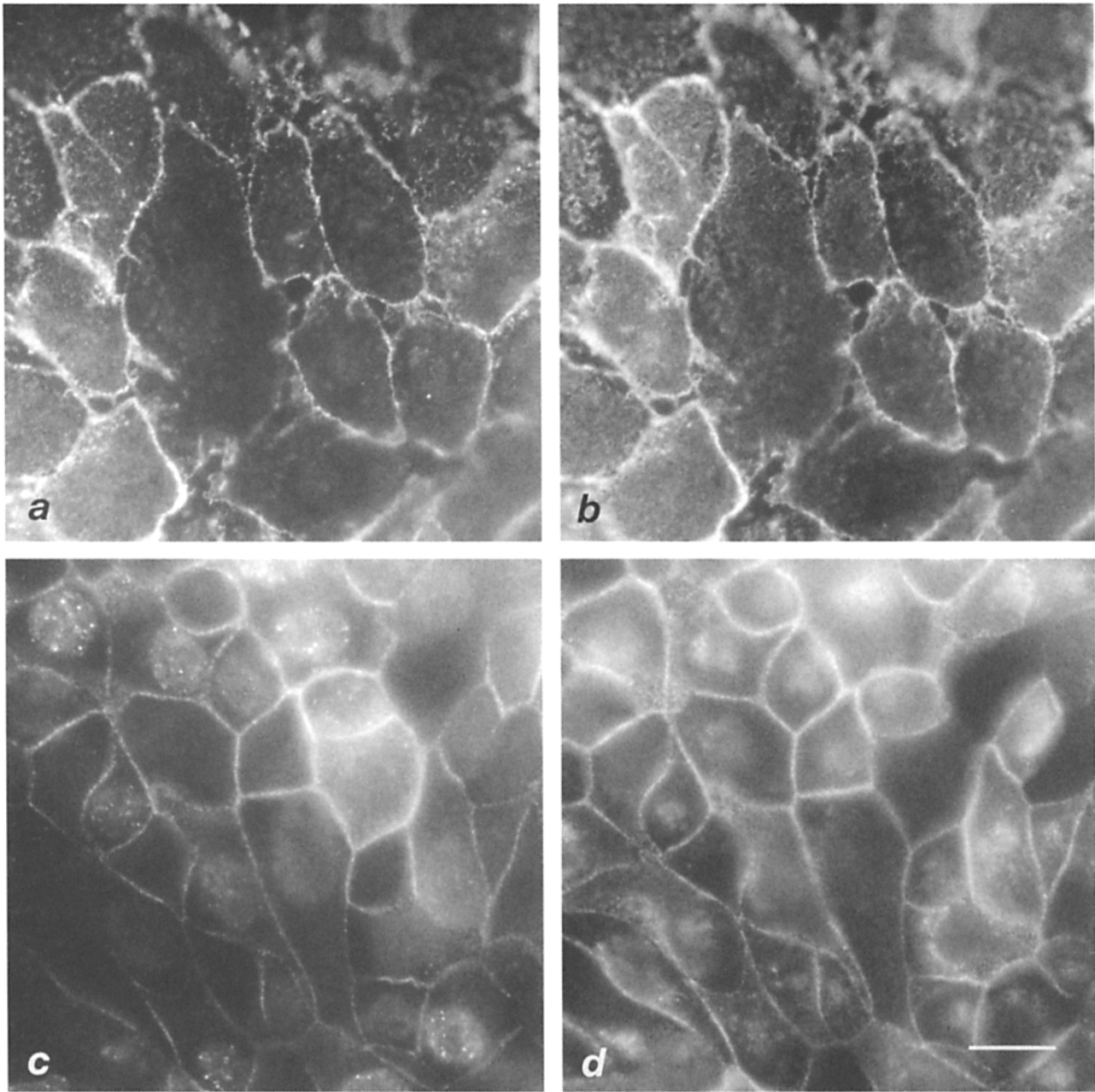
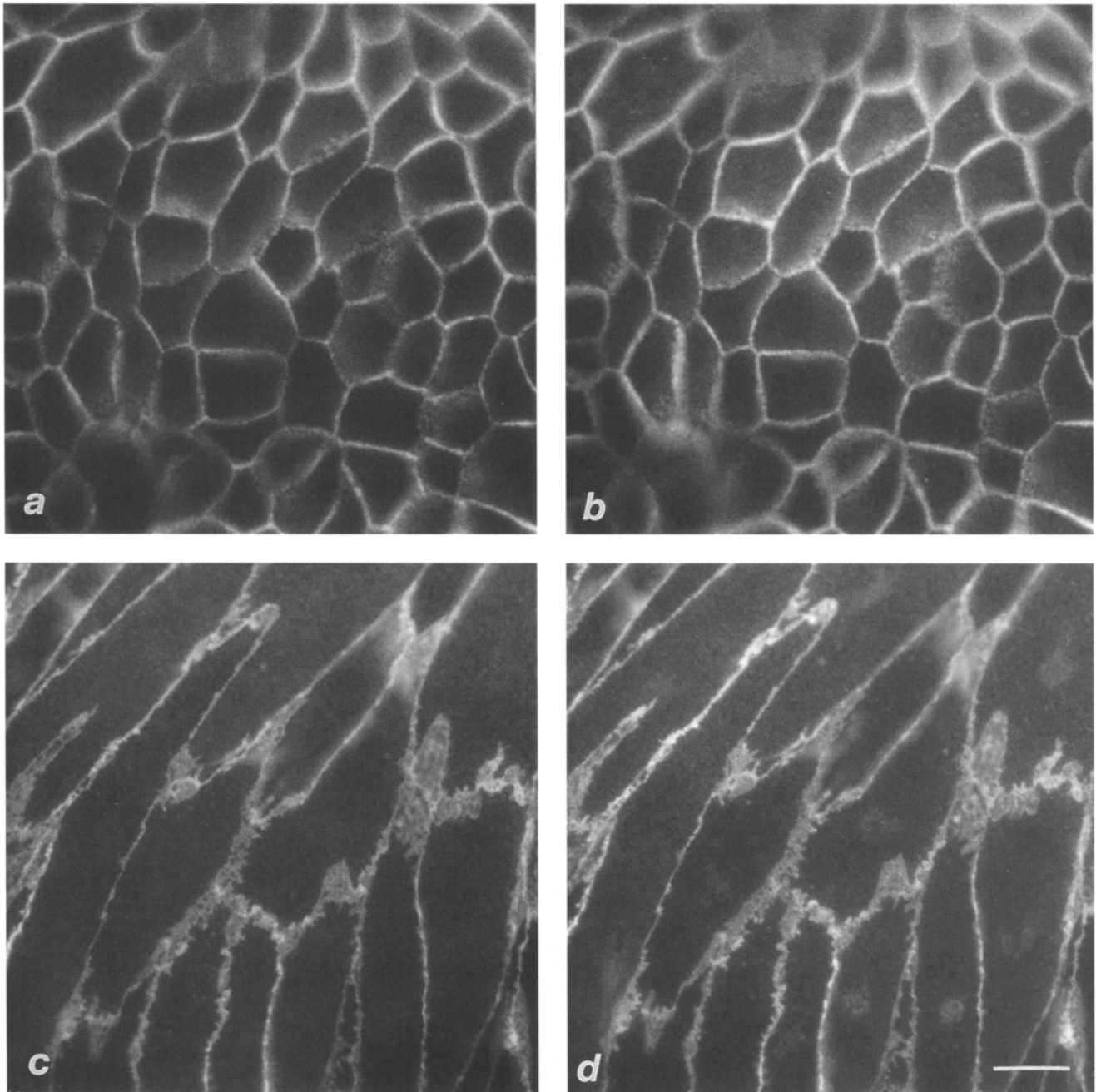*Figure 7.* Localization of p120 and β-catenin in epithelial cells. CMT 93/69 cells (*a* and *b*) and MDCK cells (*c* and *d*) were colabeled with 2B12 (*a* and *c*) and anti–β-catenin antibody (*b* and *d*). Cells were labeled after digitonin permeabilization. Secondary antibodies were fluorescein-conjugated anti-mouse and rhodamine-conjugated anti-rabbit. In this instance, it was verified that each secondary antibody was absolutely specific for its designated species of primary antibody. Bar, 20 μm.

and fluorography, the bands detected (result not shown) were similar to those presented in Fig. 3 *B*. The relative [^{35}S]methionine content of the proteins, by densitometry, gave a ratio of β-catenin:p120/p100 of 1:0.45. The half-lives (see Fig. 5) of β-catenin (13.7 h) and E-cadherin (12.0 h) were very similar (see also McCrea and Gumbiner, 1991) but shorter than that of p120/p100 (20.1 h). Therefore, correcting for the differential kinetics of protein labeling gives β-catenin:p120/p100 as 1:0.59. Correction for differences in efficiency of the immunoprecipitations was unnecessary because it was very similar (∼80%; see later) for the two antibodies. With respect to methionine content,

β-catenin contains 30 methionines (see murine [Butz et al., 1992] and *Xenopus laevis* sequence [McCrea et al., 1991]), whereas murine p120 contains 17 (Reynolds et al., 1992). Assuming that the methionine content of the canine proteins are the same, and that the methionine content of p100 is the same as that of p120, we finally get a corrected relative amount of β-catenin:p120/p100 as 1:1.04.

To determine the relative amount of p120/p100 in the cadherin/catenin complex, the cells were lysed in TX buffer, and the amount of p120/p100 in a β-catenin immunoprecipitate was analyzed (Fig. 6, *A*). Immunoblotting was used to detect protein because of the close migration

Figure 8. Localization of anti-p120 reactivity and β-catenin in MDCK cells and brain endothelial cells. MDCK cells (*a* and *b*) and porcine brain endothelial cells (*c* and *d*) were colabeled with anti-p120 antibody (*a* and *c*) and anti–β-catenin antibody (*b* and *d*). Cells were labeled after paraformaldehyde fixation. Secondary antibodies were fluorescein-conjugated anti-mouse and rhodamine-conjugated anti-rabbit. In this instance, it was verified that each secondary antibody was absolutely specific for its designated species of primary antibody. Bar, 20 μm.

of the catenins with p120/p100. For quantitation, linearity of the signal with respect to the amount of blotted protein could not be assumed; therefore, calibration curves were constructed (Fig. 6, *A* [*right*], *B,* and *C*). Furthermore, the efficiency of the immunoprecipitations had to be determined (Fig. 6 *A, left*). In the β-catenin immunoprecipitate, which was 80% efficient, 17% of the extracted pool of p120/p100 was coimmunoprecipitated. Normalizing to 100% efficiency gives 21% of the pool of p120/p100 in association with β-catenin. In the p120/p100 immunoprecipitate,

which was 83% efficient, 20% of the extracted pool of β-catenin was immunoprecipitated. Normalizing to 100% efficiency gives 24% of the pool of β-catenin in association with p120/p100. Thus, relative to β-catenin, p120/p100 is substoichiometric.

Localization to Cell–Cell Junctions

To investigate the cellular localization of p120, immunocytochemical analysis was performed. Initially, we attempted
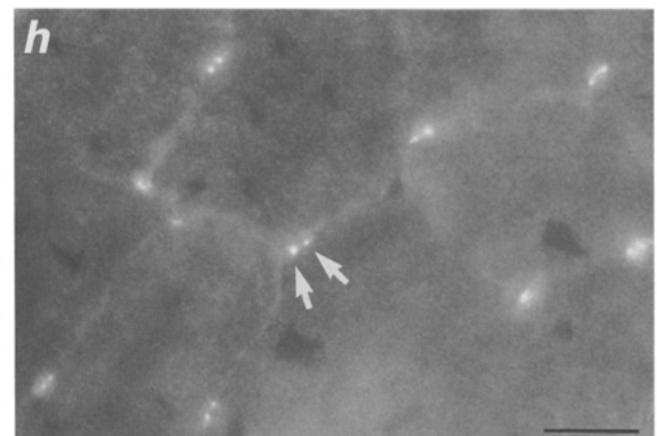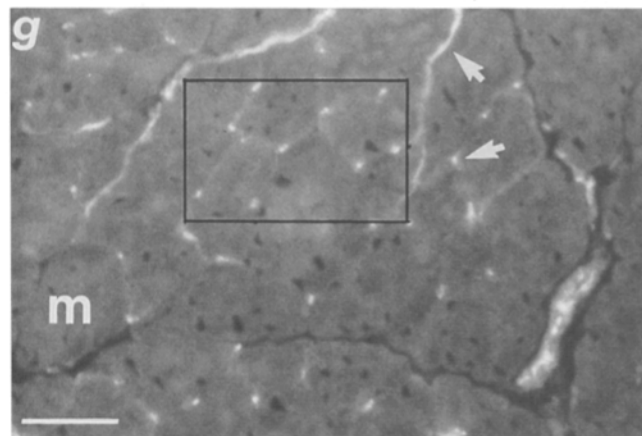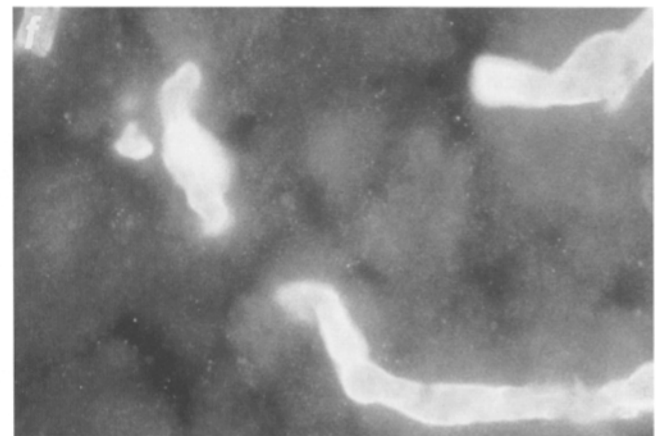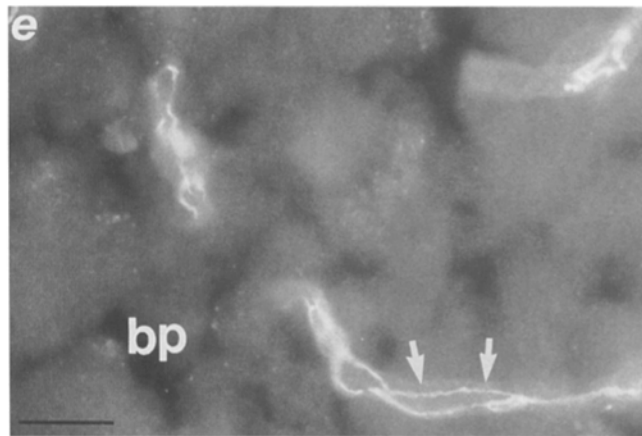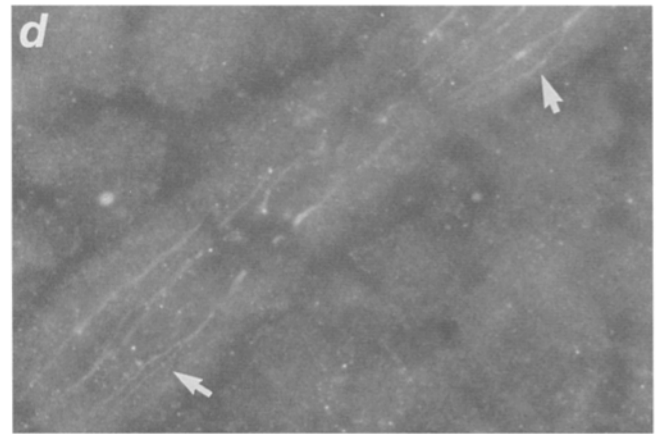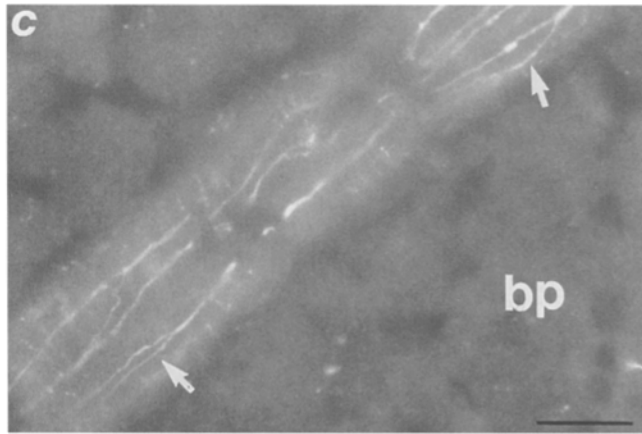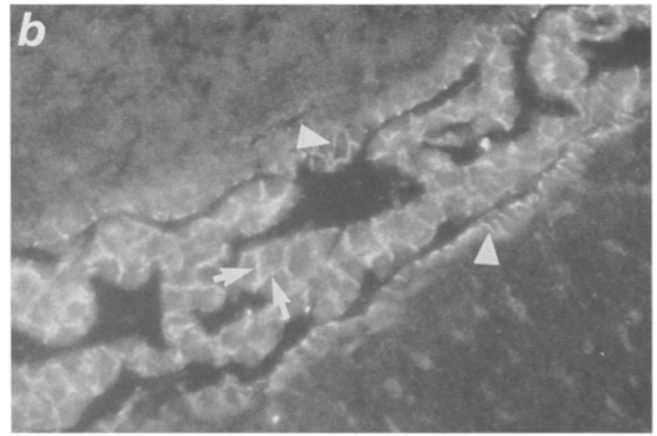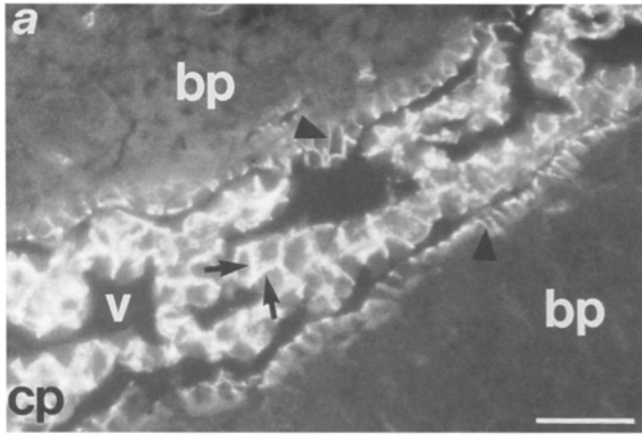
to label paraformaldehyde- or methanol-fixed MDCK cells with the 2B12 antibody, which should be specific for p120. However, in comparison with p120 from other species, 2B12 does not appear to react well with canine protein by immunoblotting (see Fig. 1 A). We therefore attempted to stain p120 in cells from these other species. Using paraformaldehyde-fixed mouse epithelial cells (which express similar amounts of p100 and p120, as detected by immunoblotting using the anti-p120 antibody; results not shown), we found that 2B12 appeared to stain intercellular junctions, but the staining was not very intense (results not shown). We then found that digitonin permeabilization of unfixed cells allowed much greater reactivity of the 2B12 antibody with its antigen, allowing the clear demonstration of colocalization of p120 (Fig. 7 a) and β-catenin (Fig. 7 b). When applied to the MDCK cells, digitonin permeabilization also revealed the colocalization of p120 (Fig. 7 c) and β-catenin (Fig. 7 d). Presumably, the 2B12 epitope is significantly masked by reactivity with formaldehyde or as a consequence of protein precipitation.

Cultured cells were also labeled with the anti-p120 antibody, which reacted well after paraformaldehyde fixation of cells. This antibody labeled MDCK (Fig. 8 a) and brain endothelial cells (Fig. 8 c) in a pattern similar to that of anti–β-catenin (Fig. 8, b and d, respectively). With the mouse epithelial cells, the labeling observed with the anti-p120 antibody after paraformaldehyde fixation was similar to that observed with the 2B12 antibody after digitonin permeabilization (results not shown). Because the anti-p120 antibody also recognizes p100, this antigen presumably has a cellular distribution similar to that of p120.

Immunocytochemistry was further performed on frozen sections of rat brain and skeletal muscle (Fig. 9). Anti-p120 immunoreactivity colocalized with α-catenin at intercellular junctions of choroid plexus epithelium and ventricular ependymal cells (Fig. 9, a and b). In addition, the anti-p120 antibody stained interendothelial junctions in both large (Fig. 9, c and d) and small blood vessels of the brain (Fig. 9, e and f) and blood vessels of muscle tissue (Fig. 9, g and h). At the given detection level, anti-p120 immunoreactivity was limited to endothelial and epithelial cells in the two types of tissue investigated.

Discussion

In the present study, immunoblot analysis of a variety of different cells revealed that the anti-p120 monoclonal antibody 2B12 (Kanner et al., 1990) recognized a broad band of ~120 kD. Another anti-p120 monoclonal antibody that had been raised against the COOH terminal portion of p120 recognized the same bands as 2B12 and, in addition,

another cluster of bands at 100 kD. The reason for the multiplicity of bands at 120 and 100 kD is not clear, but obviously they are immunologically related. The pattern of appearance of these multiple bands also depended on the cell type. For example, in MDCK cells, the bands were very diffuse, whereas in MDBK cells they were clearly resolved, especially when visualized by [35S]methionine labeling in the absence of fluorographic reagent. Enzymatic dephosphorylation of protein prior to electrophoresis did not alter the mobility of the bands, but it is still possible that other posttranslational modification, such as differential myristoylation, may give rise to mobility shifts. Apart from sharing an epitope recognized by the anti-p120 antibody, our sequence information indicates that p100 is highly related to p120. Essentially, the sequences we determined are found in three of the arm repeats of p120 (see Peifer et al., 1994), clearly establishing p100 as an arm protein. Furthermore, the Northern blots described by Reynolds et al. (1992) suggested the possibility of p120-related gene products or alternatively spliced transcripts, and Southern analysis apparently indicated that one or more p120-related genes exist (Reynolds et al., 1992). It follows that the cluster of bands corresponding to p120 and the similar cluster corresponding to p100 could also represent isoforms of p100 and p120, respectively. It is also possible that p100 could simply represent a degradation product of p120, although samples were prepared in denaturing buffer, and immunoprecipitations were performed in the presence of inhibitors of a broad spectrum of proteases. Reynolds et al. (1994) have also reported a protein of 100 kD, which they showed was related to p120 by Cleveland mapping.

Next we demonstrated that p120/p100 is associated with the cadherin/catenin complex. Our study confirms and extends the similar findings of Reynolds et al. (1994). Here, in a variety of endothelial cells, p120/p100 were shown to associate with catenins (Fig. 2) and higher molecular weight proteins (detected by [35S]methionine labeling) that probably represent cadherins (results not shown). Furthermore, we also demonstrated that independent complexes of catenins exist with p120 and p100. This apparent association between p120/p100 and the cadherin/catenin complex was not restricted to endothelial cells, because results similar to those of Reynolds et al. (1994) were obtained using epithelial cells, both by [35S]methionine labeling and immunoblot analysis (Fig. 3). We also provided an adequate explanation as to why bands corresponding to p120/p100 cannot be seen in p120/p100 immunoprecipitates from [35S]methionine-labeled, Triton-lysed MDCK cells.

The interpretation of the biochemical analyses of the protein complexes in the epithelial and endothelial cells is

Figure 9. Distribution of anti-p120 immunoreactivity in brain and skeletal muscle tissue of the rat. Brain tissue was colabeled with the anti-p120 antibody (*a* and *c*) and anti–α-catenin (*b* and *d*). Anti-p120 immunoreactivity and α-catenin colocalize at intercellular junctions of choroid plexus epithelium (*arrows, a* and *b*) and ventricular ependymal cells (*arrowheads, a* and *b*). Both antigens also colocalize at interendothelial junctions of blood vessels of macrovascular origin (*arrows, c* and *d*). Microvascular profiles in brain sections were identified by labeling with anti-collagen IV antibody (*f*); colabeling with the anti-p120 antibody (*e*) revealed the presence of antigen at interendothelial junctions (*e, arrows*). In these microvessels, α-catenin colocalized with anti-p120 immunoreactivity (not shown). In muscle tissue that had been cut perpendicular to the orientation of the muscle fibers, anti-p120 immunoreactivity is limited to areas between muscle fibers where blood vessels are located (*g, arrows*). Higher magnification reveals a punctate staining pattern (*h, arrows*), which is likely to reflect anti-p120 immunoreactivity at interendothelial junctions. *bp*, brain parenchyma; *cp*, choroid plexus; *m*, muscle tissue; *v*, ventricular lumen. Bars: (*a* and *g*) 100 μm; (*c, e,* and *h*) 25 μm.

further supported by the immunocytochemical results. The present study, and that of Reynolds et al. (1994), demonstrated junctional colocalization of anti-p120 immunoreactivity, reflecting the localization of either p120 or p100, or both, and β-catenin labeling in MDCK cells (Fig. 8). We also showed a similar, perhaps more striking, colocalization in endothelial cells (Fig. 8). Colocalization of p120 itself with β-catenin was established in our study by employing digitonin-permeabilized, unfixed cells to successfully label p120 with the p120-specific antibody 2B12 (Fig. 7). Finally, in tissue sections, the anti-p120 antibody, like the anti–α-catenin antibody, also labeled junctions between epithelial and endothelial cells (Fig. 9).

We also demonstrated that β-catenin and p120/p100 appear to be expressed to similar extents in MDCK cells, suggesting that these proteins may be organized stoichiometrically in a complex. However, the amount of p120/p100 in the cadherin/catenin complex is approximately one-fifth the amount of β-catenin. We cannot be sure if this reflects the stoichiometry in the intact cell or if the lysis conditions result in partial dissociation. Staining cells with the anti-p120 antibody reveals a mainly junctional localization of p120/p100; however, we probably would not have detected a diffusely distributed pool of protein. It is also possible that the degree of association between catenins and p120/p100 may be subject to regulation by, for example, phosphorylation. Modulation of p120/p100 association could influence the adhesiveness of cadherins or the function of catenins. Although the half-lives of β-catenin and E-cadherin were very similar, suggesting that they may be assembled and degraded as a complex (see McCrea and Gumbiner, 1991), that of p120/p100 was greater (by about half as much again), indicating that it may be independently synthesized, assembled into, and disassembled from the cadherin/β-catenin complex.

The interaction between p120/p100 and adherens junction proteins, perhaps via the influence of such regulatory kinases as src, lyn, and yes (see Tsukita et al., 1991), may play a role in the modulation of cadherin function, and thereby other cellular functions influenced by the adherens junction. With respect to phosphorylation, the tyrosine phosphatase inhibitor phenylarsine oxide was found to cause an increase in tight junction permeability in MDCK cells and brain endothelial cells and caused an increase in the tyrosine phosphorylation of junctional proteins, including β-catenin and ZO-1 (see Staddon et al., 1995). This inhibitor also increased the tyrosine phosphorylation of the anti-p120 immunoreactive material (a major p100 band, a minor p120 band) in MDCK cells, as analyzed by anti-p120 immunoblotting of anti-p120 immunoprecipitates from SDS lysates (results not shown; see Staddon et al., 1995). Reynolds et al. (1994) also examined the consequences of src-catalyzed tyrosine phosphorylation on the composition of the cadherin/catenin/p120 complex but did not detect any changes.

The p120/p100 proteins could be involved in the interaction between cadherins and the actin-based cytoskeleton. Therefore, they may also be part of a signaling cascade, communicating information about the state of cell–cell adhesiveness to the interior of the cell. Our data (Fig. 2 C) also clearly reveal separate complexes of catenins with p120 and p100. These different complexes may have differential effects on the function of the cadherin/catenin complex with respect to cell–cell adhesiveness or signaling. Furthermore, the pool of p120/p100 not associated with the cadherin/catenin complex raises the possibility that p120/p100 binds to other proteins, affecting processes other than those initiated by cadherins.

β-catenin is an arm protein (McCrea et al., 1991) and can associate with cadherins and the APC gene product (Rubinfeld et al., 1993; Su et al., 1993), also an arm protein (see Peifer et al., 1994). p120 is an arm protein as well (Reynolds et al., 1992; Peifer et al., 1994), and, as we describe here, p100 is a highly related arm protein. These proteins can interact with β-catenin. The exact nature of the interaction between p120/p100 and the catenins remains to be established. These proteins may interact directly or may associate with different regions of the cytoplasmic domain of cadherins. Other linking or intermediary binding proteins could also be involved. Clearly, there appears to be diverse interactions among arm proteins, suggesting the importance of the arm motif in intracellular signaling. Given the important role of the cadherin/catenin complex in cellular transformation and the identification of p120 as a pp60^{src} substrate, this suggests that p120/p100 may play a role in cellular growth control and other processes, such as tight junction permeability control, via an influence on cell–cell adhesion.

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