

Association of p120, a Tyrosine Kinase Substrate, with E-Cadherin/Catenin Complexes

Sayumi Shibamoto,* Makio Hayakawa,* Kenji Takeuchi,* Takamitsu Hori,* Keiji Miyazawa,‡ Naomi Kitamura,‡ Keith R. Johnson,§ Margaret J. Wheelock,§ Norihisa Matsuyoshi,|| Masatoshi Takeichi,† and Fumiaki Ito*

*Department of Biochemistry, Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata, Osaka 573-01, Japan; †Institute for Liver Research, Kansai Medical University, Moriguchi, Osaka 570, Japan; §Department of Biology, University of Toledo, Toledo, OH 43606; ||Department of Dermatology, Faculty of Medicine, Kyoto University, Shogoin, Sakyo-ku, Kyoto 606-11; and ‡Department of Biophysics, Faculty of Science, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto 606-01, Japan

Abstract. p120 was originally identified as a substrate of pp60^{src} and several receptor tyrosine kinases, but its function is not known. Recent studies revealed that this protein shows homology to a group of proteins, β -catenin/Armadillo and plakoglobin (γ -catenin), which are associated with the cell adhesion molecules cadherins. In this study, we examined whether p120 is associated with E-cadherin using the human carcinoma cell line HT29, as well as other cell lines, which express both of these proteins. When proteins that copurified with E-cadherin were analyzed, not only α -catenin, β -catenin, and plakoglobin but also p120 were detected. Conversely, immunoprecipitates of p120 contained E-cadherin and all the catenins, although a large subpopulation of p120 was not associated with E-cadherin. Analysis of these immunoprecipitates suggests that 20% or less of the extractable E-cadherin is associated with p120. When p120 immunoprecipitation was performed with cell lysates depleted of E-cadherin, β -catenin was no longer coprecipitated, and the amount of plakoglobin copurified was greatly re-

duced. This finding suggests that there are various forms of p120 complexes, including p120/E-cadherin/ β -catenin and p120/E-cadherin/plakoglobin complexes; this association profile contrasts with the mutually exclusive association of β -catenin and plakoglobin with cadherins. When the COOH-terminal catenin binding site was truncated from E-cadherin, not only β -catenin but also p120 did not coprecipitate with this mutated E-cadherin. Immunocytological studies showed that p120 colocalized with E-cadherin at cell-cell contact sites, even after non-ionic detergent extraction. Treatment of cells with hepatocyte growth factor/scatter factor altered the level of tyrosine phosphorylation of p120 as well as of β -catenin and plakoglobin. These results suggest that p120 associates with E-cadherin at its COOH-terminal region, but the mechanism for this association differs from that for the association of β -catenin and plakoglobin with E-cadherin, and thus, that p120, whose function could be modulated by growth factors, may play a unique role in regulation of the cadherin-catenin adhesion system.

THE protein termed p120 was originally identified as a major target for the oncogenic Src tyrosine kinase (Reynolds et al., 1989; Kanner et al., 1990). Among many targets of Src, p120 shows tyrosine phosphorylation best correlated with the transformation of cells, suggesting a possible link between this phosphorylation and cellular transformation. p120 also can be phosphorylated when cells are treated with several growth factors, such as EGF, PDGF, and colony-stimulating factor 1 (CSF-1),¹ whose receptors

contain tyrosine kinase domains (Downing and Reynolds, 1991; Kanner et al., 1991). Sequence analysis of p120 revealed that it has significant homology to members of the β -catenin/Armadillo gene family (Reynolds et al., 1992; Peifer et al., 1994a). β -Catenin, one member of this small family of proteins, associates with the cadherin cell-cell adhesion molecules. Plakoglobin is another member of this family (McCrea et al., 1991) and is also associated with the cadherins (Knudsen and Wheelock, 1992; Piepenhagen and Nelson, 1993). Armadillo is a *Drosophila* homologue of β -catenin; our recent study demonstrated that this protein forms a complex with a newly identified *Drosophila* cadherin (Oda et al., 1994). Thus, all of these molecules are cadherin-associated proteins. However, it has not been determined whether p120 also binds to cadherins.

Address all correspondence to Masatoshi Takeichi, Department of Biophysics, Faculty of Science, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto 606-01, Japan. Ph.: (81) 75 753 4196. Fax: (81) 75 753 4197.

1. *Abbreviations used in this paper:* AJ, adherens junctions; CSF-1, colony-stimulating factor 1; HGF/SF, hepatocyte growth factor/scatter factor.

Cadherins are a group of cell-cell adhesion molecules constituting a superfamily (Takeichi, 1993). The "classic" cadherin subfamily, composed of such proteins as E-, P-, and N-cadherin, is known to play a fundamental role in cell-cell associations (Takeichi, 1991). These molecules are concentrated in intercellular adherens junctions (AJ), forming a molecular complex not only with β -catenin or plakoglobin but also with α -catenin (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989; McCrea et al., 1991; Knudsen and Wheelock, 1992; Peifer et al., 1992; Piepenhagen and Nelson, 1993). This molecular complex further interacts with a cluster of other cytoskeletal proteins, including vinculin, α -actinin, fodrin, and actin bundles. The cadherin-associated proteins are visualized ultrastructurally as the cytoplasmic plaque or undercoat of AJ (Hirano et al., 1987; Nelson et al., 1990; Shore and Nelson, 1991; McNeill et al., 1993). As demonstrated by several experiments, association with these cytoplasmic proteins is crucial for the activity of cadherins (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989; Hirano et al., 1992; Shimoyama et al., 1992). For example, α -catenin-deficient PC9 cells cannot aggregate in a cadherin-dependent manner despite their expression of E-cadherin and β -catenin (Hirano et al., 1992; Shimoyama et al., 1992). In addition, a recent study demonstrated that a cell line with an NH₂ terminally truncated β -catenin cannot aggregate although the cells express α -catenin and E-cadherin (Oyama et al., 1994).

The cytoplasmic plaque of AJ contains members of the Src family, such as Src, Yes, and Lyn (Rohrschneider, 1980; Tsukita et al., 1991). Recent studies demonstrated that v-Src-mediated transformation of cells induces strong phosphorylation of β -catenin and, depending upon the cell line, also weak phosphorylation of α -catenin and E-cadherin (Matsuyoshi et al., 1992; Hamaguchi et al., 1993; Behrens et al., 1993). Hepatocyte growth factor/scatter factor (HGF/SF) and EGF can also induce or enhance tyrosine phosphorylation of not only β -catenin but also plakoglobin in cells which can respond to these growth factors (Shibamoto et al., 1994). Interestingly, the enhancement of tyrosine phosphorylation of these proteins is correlated with perturbation of cadherin activity. For example, N-cadherin activity is blocked in chicken fibroblasts transformed with Rous sarcoma virus, where strong phosphorylation of these proteins is observed (Hamaguchi et al., 1993). In 3Y1 cells transformed with v-src, where only β -catenin is phosphorylated, cadherin-mediated cell-cell contacts are unstable (Matsuyoshi et al., 1992). Likewise, HGF/SF and EGF are known to induce scattering of certain epithelial cells (Stoker et al., 1987; Weidner et al., 1990; Shibamoto et al., 1994). These observations suggest that tyrosine phosphorylation of catenins/plakoglobin may affect the activity of the cadherin adhesion system.

It should also be noted that Armadillo, the *Drosophila* β -catenin homologue, is a segment polarity gene product (Peifer and Wieschaus, 1990; McCrea et al., 1991); that is, this protein plays a role in the determination of cell fates during the organization of segment polarity in *Drosophila* embryos. The activity of Armadillo is downstream of several other genes, including *wingless*, *dishevelled*, and *zeste-white 3* (Peifer et al., 1993; Siegfried et al., 1994; Noordermeer et al., 1994; Peifer et al., 1994b). It is thus postulated that Armadillo may function as a signaling molecule to control cell interactions and differentiation. A recent study demonstrated that injection of antibodies against β -catenin into

Xenopus eggs induced double body axis formation (McCrea et al., 1993), thus implicating it in cellular differentiation. Another important finding was that β -catenin and plakoglobin are associated with the product of APC, a tumor suppressor gene, whose mutations have been implicated in familial adenomatous polyposis as well as sporadic colorectal cancer (Su et al., 1993; Rubinfeld et al., 1993; Shibata et al., 1994). These findings suggest that members of the β -catenin/Armadillo family play a signaling role in both development and oncogenesis, and that their tyrosine phosphorylation could be one of the steps necessary for such a signaling process. The correlation between the tyrosine phosphorylation of p120 and the Src-mediated transformation of cells suggests that p120 may be involved in such a process.

In the present study, we examined whether p120 is associated with E-cadherin or catenins, and whether tyrosine phosphorylation of p120 is altered by HGF/SF treatment. The results show that p120 is indeed associated with E-cadherin but in a way different from that of β -catenin and plakoglobin, and that phosphorylation on tyrosine is affected by HGF/SF treatment. These findings suggest that p120 is involved in regulation of the cadherin-catenin adhesion apparatus or in signaling via this adhesion system.

Materials and Methods

Immunoreagents and Hepatocyte Growth Factor/Scatter Factor

Mouse mAb HECD-1 to human E-cadherin (Shimoyama et al., 1989), rat mAb ECCD-2 to mouse E-cadherin (Shirayoshi et al., 1986), a rabbit antiserum to mouse E-cadherin (Nagafuchi et al., 1987), a mouse mAb to p120 (Transduction Laboratories, Lexington, KY), and mouse mAb PY20 to phosphotyrosine (ICN ImmunoBiologicals, Costa Mesa, CA) were used. Rat mAb α -18 to α -catenin (Nagafuchi and Tsukita, 1994) was kindly provided by Akira Nagafuchi (Kyoto University, Kyoto, Japan). A polyclonal antiserum to β -catenin was raised by immunizing a rabbit with a KLH conjugated to a peptide corresponding to the COOH-terminal 14 amino acids of β -catenin. A mouse mAb, 15F11, was prepared against human plakoglobin. A restriction fragment spanning the entire open reading frame of plakoglobin was isolated from a cDNA clone, kindly provided by Dr. Werner Franke (German Cancer Research Center, Heidelberg, FRG), and inserted into the pMAL-c2 expression vector (New England Biolabs, Beverly, MA). The production of mAb 15F11 was carried out as previously described (Johnson et al., 1993).

For detection of primary antibodies, the following secondary antibodies were used: Sheep HRP-conjugated anti-mouse Ig antibody, sheep biotinylated anti-mouse Ig antibody, sheep HRP-conjugated anti-rat Ig antibody, and FITC-labeled streptavidin (Amersham, Buckinghamshire, UK); swine HRP-linked anti-rabbit Ig antibody (DAKOPATTS, Glostrup, Denmark); and goat Texas red-conjugated anti-rabbit IgG antibody (CAPPEL Research Products, Durham, NC).

The human HGF/SF was a recombinant protein produced in Chinese hamster ovary cells as described previously (Miyazawa et al., 1989; Strain et al., 1991).

Cells and cDNA Transfection

HT29 cells (ATCC-HTB38) were cultured in DME supplemented with 10% FCS. A line of Ras-transformed NIH3T3 cells, kindly provided by Dr. Tadashi Yamamoto (University of Tokyo, Tokyo, Japan), was transfected with pBATEM2 encoding the intact mouse E-cadherin (Nose et al., 1988) or with pBATEM21 encoding a mutant E-cadherin whose carboxy terminal 37 amino acids were deleted (Nagafuchi and Takeichi, 1989). These transfectants were cultured in the same medium as above.

Preparation of Cellular Extracts

Cells were seeded at the density of 1×10^6 cells per 94-mm dish in DME with 5% FCS and incubated for 3 d. The cultures were then washed with a Hepes-buffered saline containing 1 mM CaCl₂ (HMF) supplemented

with 1 mM Na₃VO₄, 3 mM H₂O₂, and a cocktail of protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 1 μg/ml *p*-toluenesulfonyl-L-arginine methyl ester, and 2 μg/ml pepstatin A). Subsequently, cells were lysed in a nonionic detergent extraction buffer (20 mM Tris-buffered saline (pH 7.4), 1% NP-40, 1% Triton X-100, 1 mM Na₃VO₄, 3 mM H₂O₂, 50 mM NaF, 30 mM Na₄P₂O₇, and the cocktail of protease inhibitors) for 30 min on ice. The crude extracts were clarified by centrifugation, and the supernatants (soluble fraction) and pellets (insoluble fraction) were separated. For examination of total cellular lysates, cells were lysed with Laemmli SDS sample buffer (Laemmli, 1970).

Immunoprecipitation and Immunoblotting

Immunoprecipitation of E-cadherin and other proteins with specific antibodies was carried out using the nonionic detergent-soluble fraction of cells, as previously described (Shibamoto et al., 1994). For complete depletion of E-cadherin from cell extracts, three cycles of immunoprecipitation were performed with HECD-1; these samples were always monitored for the absence of residual E-cadherin. The "preabsorbed" extracts were used for subsequent immunoprecipitation of other proteins. Proteins in immunoprecipitates were resolved by 7.5% SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA). The membranes were incubated with primary antibodies for 2 h, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h, subsequently developed with the Enhanced Chemiluminescence Reagent (ECL, Amersham, UK), and finally exposed to Kodak X-OMAT AR film.

To collect radiolabeled immunoprecipitates, cells were incubated with 100 μCi/ml of [³⁵S]methionine (1,000 Ci/mmol; NEN Research Products, Boston, MA) in methionine-free DME with 5% FCS for 15 h. Immunoprecipitation of proteins from these cells was performed as described above. The radiolabeled immunoprecipitates were then immunoblotted, and the filters were used for autoradiography.

Immunocytochemistry

Double-immunofluorescence staining for E-cadherin and p120 was carried out as follows. Cells were cultured on glass coverslips and fixed with 3.5% paraformaldehyde in HMF for 30 min on ice. For some experiments, cells were treated with the detergent extraction buffer for 30 min prior to paraformaldehyde fixation. The fixed cells were further treated with methanol at -20°C for 15 min, and blocked with 5% skim milk for 30 min. Cells were incubated with a mixture of the rabbit anti-E-cadherin antiserum and mouse anti-p120 mAb for 60 min, followed by incubation with biotinylated species-specific antibodies to mouse Ig for 30 min, and finally incubated with FITC-labeled streptavidin and Texas red-conjugated species-specific antibodies to rabbit IgG. These samples were examined using a Zeiss Axiophot microscope.

Results

Detergent Extractability of p120, E-Cadherin, and Catenins

HT29 cells expressed p120 as well as E-cadherin and its associated proteins, β-catenin, and plakoglobin. E-Cadherin was the major cadherin molecule expressed by this cell line, so only this cadherin was studied. We first examined detergent solubility of these proteins by extracting cells with a solution containing the nonionic detergents NP-40 and Triton X-100. The soluble and insoluble materials were resolved by SDS-PAGE and analyzed by immunoblotting. While all of the proteins were present in both the soluble and insoluble fractions, the proportion of individual proteins in the two fractions varied. For E-cadherin and β-catenin, the soluble fraction contained a little more than the insoluble fraction (Fig. 1, *E-cad*, *β-cat*). Slightly more plakoglobin was in the insoluble fraction (Fig. 1, *plak*), probably because this protein is associated with nonionic detergent-resistant desmosomes (Cowin et al., 1986). Compared with these proteins, the ratio of p120 in the soluble fraction vs. the insoluble fraction was much greater (Fig. 1, *p120*); the majority of this molecule was detected in the soluble fraction.

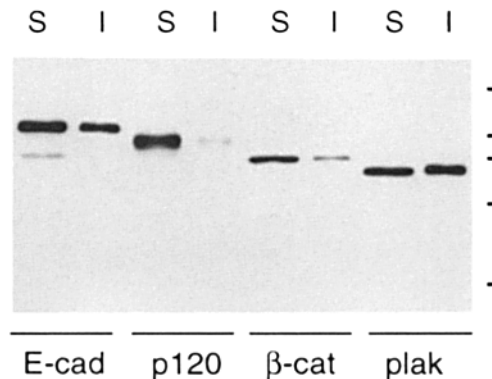


Figure 1. Immunoblot analysis of detergent soluble and insoluble fractions of HT29 cells. The nonionic detergent-soluble (S) and -insoluble (I) fractions were prepared as described in Materials and Methods, dissolved with the same amount of SDS sample buffer, and subjected to immunoblot analysis for detection of E-cadherin, p120, β-catenin, and plakoglobin. Molecular weight markers for 200, 116, 97.4, 66.2, and 45 × 10³ are indicated by bars.

Association of p120 with E-Cadherin and Catenins

Previous reports have shown that E-cadherin coimmunoprecipitates with α-catenin, β-catenin, and plakoglobin (γ-catenin) from metabolically labeled cell lysates. We prepared nonionic detergent extracts of HT29 cells labeled with [³⁵S]-methionine, and collected immunoprecipitates of E-cadherin. Electrophoretic analysis of these samples confirmed the coprecipitation of E-cadherin with α-catenin, β-catenin, and plakoglobin, and also detected very faint bands at the 110–115-kD region (Fig. 2 A, lanes 1 and 2). We then analyzed p120 immunoprecipitates prepared from ³⁵S-labeled HT29 cells, and found that this protein coprecipitated with at least four bands, which comigrated with E-cadherin, α-catenin, β-catenin, and plakoglobin, respectively (Fig. 2 A, lanes 3 and 4).

We then prepared non-radiolabeled E-cadherin and p120 immunoprecipitates, and analyzed them by immunoblotting for associated proteins (Fig. 2 B). In E-cadherin immunoprecipitates, not only β-catenin and plakoglobin but also p120 was detected. In reverse experiments using p120 immunoprecipitates, E-cadherin was found to coprecipitate with p120. In the p120 immunoprecipitates, β-catenin, and plakoglobin were also observed. We also analyzed material immunoprecipitated with anti-β-catenin or anti-plakoglobin antibodies. In β-catenin immunoprecipitates, neither p120 nor plakoglobin was detected while β-catenin and a small amount of p120 were detected in plakoglobin immunoprecipitates. Both immunoprecipitates contained E-cadherin, as expected. All of these results were reproducible in repeated experiments. Thus, p120 is associated with E-cadherin, although it is not a major component in the E-cadherin immunoprecipitates, and p120 is also coprecipitable with β-catenin and plakoglobin.

Next, we attempted to assess the fraction of both p120 and E-cadherin that are in the p120/E-cadherin complex. p120 and E-cadherin immunoprecipitates were collected from aliquots of the same cell extract, sequentially diluted and blotted onto a single sheet of polyvinylidene difluoride membrane after SDS-PAGE. This sheet was probed sequentially to detect p120 and E-cadherin. As shown in Fig. 3, more p120 was recovered from the immunoprecipitation using

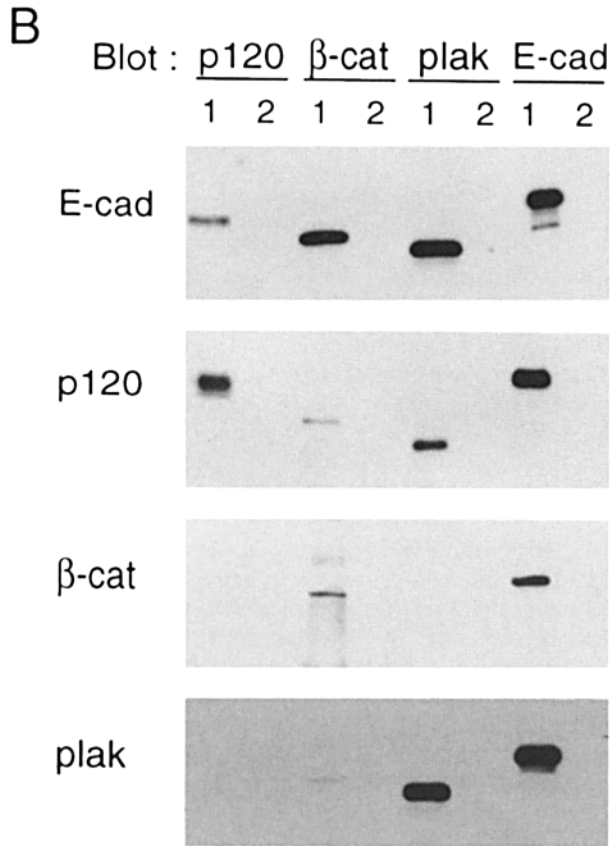
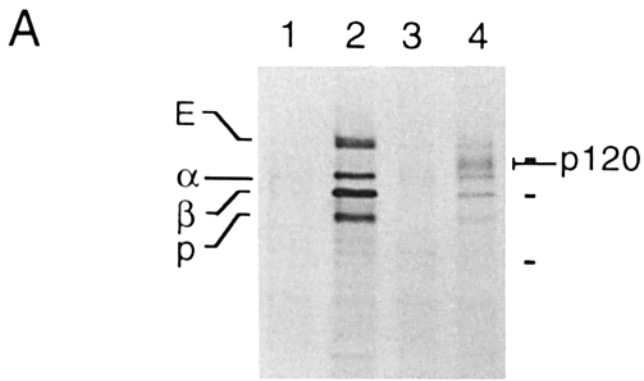


Figure 2. Analysis of proteins copurified with E-cadherin, p120, β -catenin, and plakoglobin. (A) Autoradiograms of radiolabeled E-cadherin immunoprecipitate (lane 2) and p120 immunoprecipitate (lane 4) after SDS-PAGE. Lanes 1 and 3 are control immunoprecipitate samples obtained with nonimmunized IgG. E, E-cadherin; α , α -catenin; β , β -catenin; p, plakoglobin. Molecular weight markers for 116, 97.4, and 66.2×10^3 are indicated by bars. (B) Immunoblot analyses of proteins coprecipitated with E-cadherin (E-cad), p120, β -catenin (β -cat), or plakoglobin (plak) (each lane 1). The sample run in each lane 2 was a control where either non-immunized mouse IgG or rabbit IgG was used for the immunoprecipitation. The lower molecular weight bands observed in some E-cadherin immunoblots may be degradation products. The higher and lower bands in the β -catenin sample may be non-specific antibody reactions.

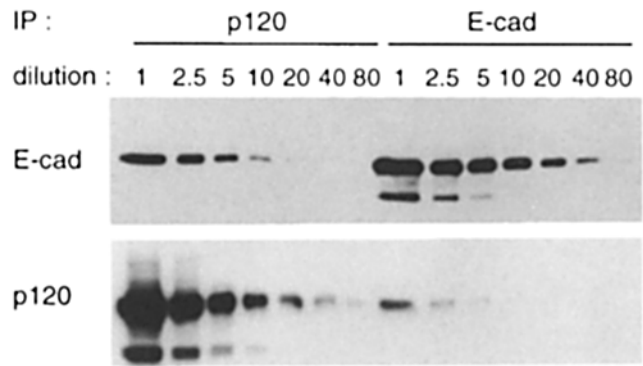


Figure 3. Stoichiometric analysis of p120/E-cadherin complexes. Immunoprecipitates of p120 and E-cadherin obtained from equal aliquots of the same cellular extract were diluted as indicated, and subjected to SDS-PAGE. After blotting proteins to a single membrane sheet, the membrane was probed sequentially for p120 and E-cadherin. By comparing the intensities of the bands between the samples, the relative amount of the p120/E-cadherin complex to the entire pool of each protein was estimated, as described in the text. IP, immunoprecipitation.

anti-p120 than from the immunoprecipitation using anti-E-cadherin. Likewise, more E-cadherin was recovered from the immunoprecipitation using anti-E-cadherin than from the immunoprecipitation using anti-p120. In these immunoprecipitations, most of the antigens were recovered from each cell lysate, as checked by immunoblotting of the depleted lysates. These results suggest that the p120/E-cadherin complex represents only part of the entire pool of each molecule. From the data of Fig. 3, we can estimate that $\sim 5\%$ of p120 was associated with E-cadherin, and $\sim 20\%$ of E-cadherin formed a complex with p120. However, in other experiments $<20\%$ of E-cadherin was complexed with p120; in one extreme case, it was only 2%. This indicates that the association of p120 with E-cadherin may be affected by unknown conditions.

We then explored how the above molecular complexes were formed. We depleted cell lysates of E-cadherin by pre-immunodepletion with anti-E-cadherin antibody, and subjected these preabsorbed samples to immunoprecipitation with anti-p120 antibody. In the resultant p120 immunoprecipitates, β -catenin was no longer present (Fig. 4 A, lane 1), and plakoglobin coprecipitation was greatly reduced (Fig. 4 B, lane 1); more than 75% of the plakoglobin originally coprecipitable with p120 (Fig. 4 B, lanes 2–5) was lost after the E-cadherin depletion. This finding suggests that there are multiple forms of p120 complexes, such as p120/E-cadherin/ β -catenin, p120/E-cadherin/plakoglobin, and p120/plakoglobin (see Discussion).

In a similar study, α -catenin was detected in p120 immunoprecipitates by immunoblot analysis (Fig. 4 C, lane 2). However, when cell lysates were depleted of E-cadherin, α -catenin no longer coprecipitated with p120 (Fig. 4 C, lane 1). This result indicates that α -catenin is associated with p120/E-cadherin complexes.

Localization of the p120 Binding Region on E-Cadherin

It is known that catenins bind to the COOH-terminal region of E-cadherin. To investigate whether the same site is in-

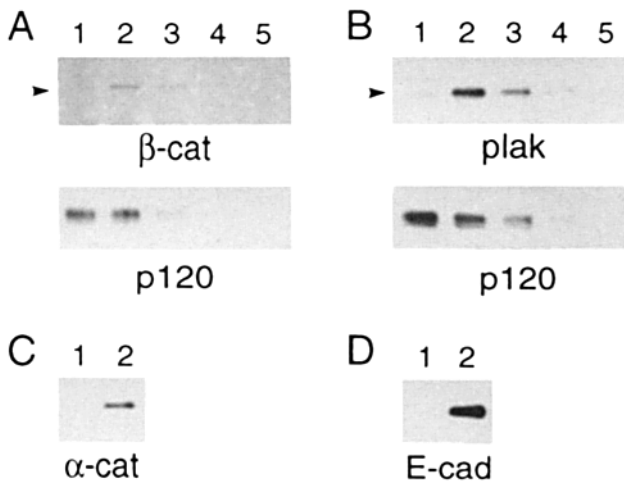


Figure 4. Effect of E-cadherin depletion on the coprecipitation of catenins with p120. p120 was immunoprecipitated from a cell lysate depleted of E-cadherin by means of preabsorption with HECD-1. β -Catenin (A, lane 1), plakoglobin (B, lane 1), or α -catenin (C, lane 1) in this immunoprecipitate was analyzed by immunoblotting. For comparison, p120 was immunoprecipitated from a non-preabsorbed cell lysate, and this p120 immunoprecipitate was sequentially diluted 1 \times (lane 2), 2 \times (lane 3), 4 \times (lane 4), and 8 \times (lane 5), and used for immunodetection of each catenin. The same filter was then reprobed with anti-p120 antibody (lower panels), although these data were omitted for (C). Arrow indicates the position of β -catenin or plakoglobin. An upper band in lane 1 of (B) was not identified. (D) Immunoblot detection of E-cadherin in the E-cadherin-depleted lysate (lane 1) and non-depleted sample (lane 2) before the p120 immunoprecipitation. Neither β -catenin nor α -catenin was detected in the E-cadherin-depleted sample. On the other hand, a small amount of plakoglobin which is equivalent to that detected in the non-absorbed sample diluted more than 4 \times was found in the E-cadherin-depleted sample.

involved in p120 association, we prepared a 3T3 cell line transfected with a cDNA encoding a COOH terminally truncated E-cadherin to which no known catenin can bind (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989), and a control 3T3 line transfected with normal E-cadherin cDNA (Fig. 5, lanes 1 and 2). From these cell lines, E-cadherin was immunoprecipitated and analyzed for p120 binding. As shown in Fig. 5 (lanes 3–10), neither β -catenin nor p120 was co-purified

with the truncated E-cadherin, whereas both coprecipitated with the intact E-cadherin. In addition, it should be noted that the levels of p120 expressed by the two cell lines were almost identical to each other (Fig. 5, lanes 11 and 12), whereas the levels of β -catenin were not identical; i.e., more β -catenin was detected in cells expressing normal E-cadherin (Fig. 5, lanes 13 and 14). These findings indicate that p120 forms complexes with E-cadherin/catenins at the COOH-terminal region of E-cadherin.

Subcellular Localization of p120

HT29 cells were double-immunostained for p120 and E-cadherin. p120 was concentrated at cell-cell contact sites and colocalized with E-cadherin (Fig. 6, a and b). We also examined the distribution of p120 after a nonionic detergent extraction of cells and found that not only E-cadherin but also p120 were still present at cell-cell contact sites, although the intensity of the staining was much reduced (Fig. 6, c and d). These data suggest that a certain population of p120 was anchored to the cytoskeleton together with E-cadherin at cell-cell contact sites. It should be noted that, in these samples, much of the p120 signal did not colocalize with E-cadherin when the focus was adjusted to non-junctional areas (data not shown).

Tyrosine Phosphorylation of p120 Is Altered by HGF

p120 is known to be a tyrosine kinase substrate. We examined whether the E-cadherin-associated p120 was tyrosine phosphorylated and whether this phosphorylation could be modified by HGF/SF in HT29 cells. Immunoblot analysis with anti-phosphotyrosine revealed that p120 was constitutively phosphorylated at tyrosine residues in this cell line (Fig. 7, top). Treatment of cells with HGF/SF for 10 min induced a strong suppression of this phosphorylation; however, the phosphorylation gradually recovered to normal levels after a 30 min incubation. A similar change in tyrosine phosphorylation was observed for a 115-kD protein that was associated with E-cadherin (Fig. 7, top), as well as for β -catenin and plakoglobin. This 115-kD protein was most likely p120, since the two proteins co-migrated on SDS-PAGE. After an overnight incubation with HGF/SF, a slight increase in the level of tyrosine phosphorylation of these proteins was observed (data not shown), as previously reported (Shibamoto et al., 1994). The time required for the suppression and recovery

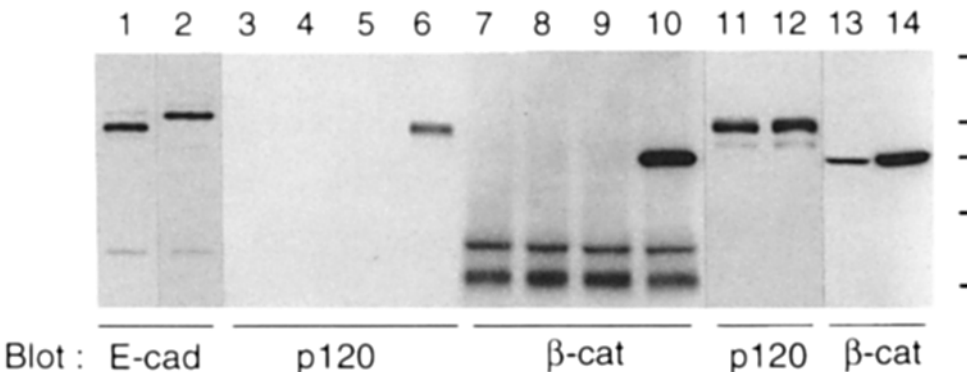


Figure 5. Association of p120 and β -catenin with intact E-cadherin or E-cadherin lacking the catenin-binding region. 3T3 cells expressing full-length E-cadherin (lanes 2, 6, 10, 12, and 14) or truncated E-cadherin lacking the catenin-binding region (lanes 1, 4, 8, 11, and 13) were immunoprecipitated with anti-E-cadherin (lanes 1, 2, 4, 6, 8, and 10) or nonimmune IgGs (lanes 3, 5, 7, and 9). Lanes 11 to 14 contain total cell lysates.

The blots were probed for E-cadherin, p120, or β -catenin as indicated. The truncated E-cadherin is a little smaller than the intact molecule. The bands observed around 55–60-kD represent rat immunoglobulin heavy chains reacting with the secondary antibodies. Molecular weight markers for 200, 116, 97.4, 66.2, and 45 $\times 10^3$ are indicated by bars.

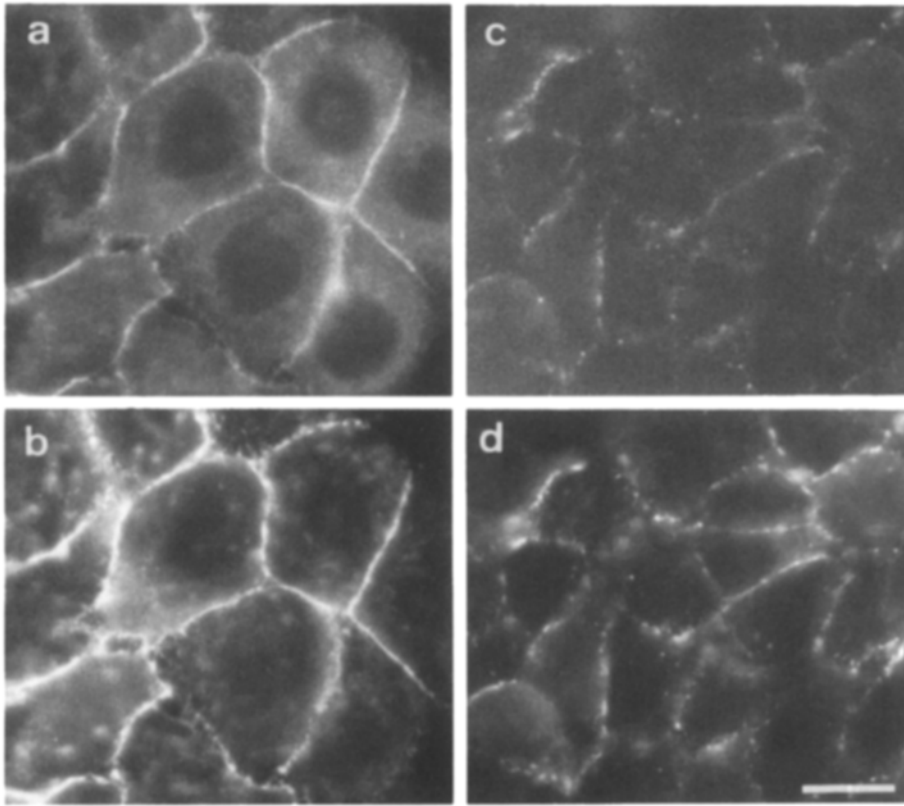


Figure 6. Immunofluorescence localization of p120 and E-cadherin in HT29 cells. Double-immunofluorescence staining for p120 (a and c) and E-cadherin (b and d). In c and d, cells were treated with the nonionic detergent mixture prior to fixation (see Materials and Methods), followed by immunofluorescence staining. Bar, 10 μ m.

of the tyrosine phosphorylation varied from experiment to experiment. We also noticed that tyrosine phosphorylation of these proteins in response to HGF/SF was somewhat altered by different culture conditions, suggesting the involvement of varying environmental factors. However, the above results were reproducible under the experimental conditions used.

Although tyrosine phosphorylation of p120 was dramatically changed after HGF/SF treatment, the level of p120 associated with E-cadherin did not change (Fig. 7, bottom). Tyrosine phosphorylation of the p120 associated with E-cadherin was also observed in other human carcinoma lines such as MKN74 cells (data not shown).

Discussion

In the present study, we demonstrated that p120 is associated with E-cadherin. Despite the distribution of p120 in a wide variety of cells, previous studies have not detected this molecular complex; probably because p120 is not a major component of cadherin immunoprecipitates, as compared with the other known catenins. The association of p120 with cadherins, however, may occur in many cell types; in fact, we have found it in two completely different cell types, a colon carcinoma cell (HT29), and a fibroblast (3T3).

Recent studies are uncovering the mechanisms by which cadherins are associated with α -catenin, β -catenin, and plakoglobin. Evidence is accumulating that β -catenin or plakoglobin directly associates with cadherins (Hinck et al., 1994; Näthke et al., 1994) and that their binding to N-cadherin is mutually exclusive (Hinck et al., 1994). It seems that the binding of β -catenin to E-cadherin occurs through the

12-repeat region. In addition, the association of α -catenin with E-cadherin is probably mediated by β -catenin or plakoglobin, as α -catenin cannot be coprecipitated with E-cadherin when NH₂ terminally truncated β -catenin is expressed in cells (Oyama et al., 1994; W. Birchmeier, personal communication). The linkage between cadherin and α -catenin via β -catenin must be crucial for this adhesion system, since α -catenin is required for cadherin function (Hirano et al., 1992; Shimoyama et al., 1992).

How, then, is p120 associated with E-cadherin? p120 was coprecipitated not only with E-cadherin but also with β -catenin and plakoglobin. The depletion of E-cadherin by pre-immunoprecipitation from cell lysates resulted in the removal of the β -catenin and a reduction of the plakoglobin that was coprecipitable with p120. This finding suggests that there exist multiple forms of p120 complexes. Since β -catenin was copurified with p120 only in the presence of E-cadherin, there should be a p120/E-cadherin/ β -catenin complex. The reduced plakoglobin/p120 coprecipitation from E-cadherin-depleted samples also suggests the existence of a p120/E-cadherin/plakoglobin complex. On the other hand, a certain amount of plakoglobin could be coprecipitated with p120 without E-cadherin, suggesting that there is a p120/plakoglobin complex. The association of p120 with E-cadherin differs from the association of β -catenin or plakoglobin with E-cadherin; the latter two are mutually exclusive, as mentioned above. There are two possible mechanisms to explain p120/E-cadherin association: One is that p120 directly binds to E-cadherin. In this case, p120 must recognize a site on E-cadherin that is distinct from that recognized by β -catenin and plakoglobin, since a single cadherin molecule can associate with both p120 and β -catenin or plakoglobin. The

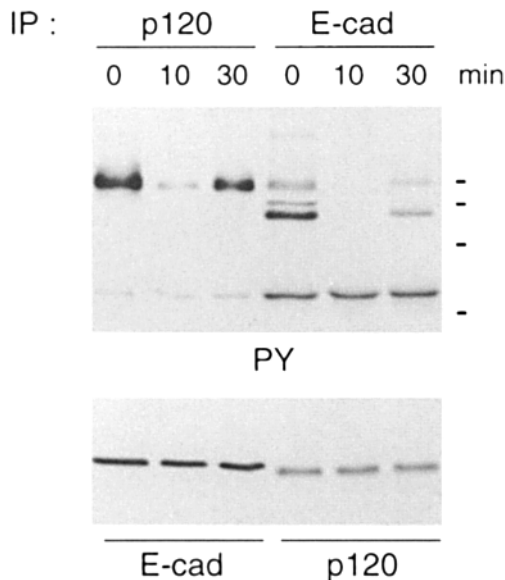


Figure 7. Detection of phosphotyrosine in p120 and E-cadherin immunoprecipitates obtained from cells treated with HGF/SF. Cells were incubated with 10 ng/ml HGF/SF for 0, 10, and 30 min at 37°C. p120 or E-cadherin was immunoprecipitated from equal aliquots of cell extract. These immunoprecipitates were resolved by SDS-PAGE, and subjected to immunoblot analysis of phosphotyrosine (PY). The E-cadherin immunoprecipitates were also probed for E-cadherin itself and p120 (*lower panel*). No changes in the amount of p120 that coprecipitated with E-cadherin were seen during the HGF/SF treatment. The tyrosine phosphorylated bands of 98- and 85-kD co-migrated with β -catenin and plakoglobin, respectively (data not shown). The bands around 55-kD represent mouse immunoglobulin heavy chains reacting with the secondary antibodies. Molecular weight markers of 116, 97.4, 66.2, and 45×10^3 are indicated by bars.

idea of direct binding of p120 to E-cadherin would be supported if we could detect p120 associated with E-cadherin in the absence of other catenins. We actually attempted to deplete cell lysates of both β -catenin and plakoglobin in search of such a p120 complex, but the complete and simultaneous depletion of these two proteins was technically difficult in our hands. The first possibility thus remains to be tested. The second possibility is that p120 binds to β -catenin or plakoglobin but not directly to E-cadherin. This is possible since β -catenin (Rubinfeld et al., 1993; Su et al., 1993) and plakoglobin (Shibata et al., 1994) have been shown to associate with APC, which is similar to p120 in the repeat motifs (Peifer et al., 1994a). We have indeed suggested that p120 can form a complex with plakoglobin, by detecting a p120/plakoglobin complex without E-cadherin. If the second possibility is true, the association profile of p120 with E-cadherin should be completely different from that of β -catenin or plakoglobin, p120 being present as an additional component of the E-cadherin/catenin complexes. We estimated the proportion of the p120-associated E-cadherin to the entire E-cadherin pool to be 20% or less in HT29 cells, indicating that only a minor fraction of the E-cadherin complexes have p120.

The association profile between β -catenin-related proteins seems to be a little more complicated. In our immunoprecipitation experiments, a small amount of β -catenin was co-

purified with plakoglobin, suggesting the presence of a complex between these two molecules, although it is a minor fraction of the entire pool. From the results of reciprocal immunoprecipitation experiments, we can estimate the relative amounts of the β -catenin-related protein complexes. The ratio of the plakoglobin/ β -catenin complex to the entire pool of plakoglobin, and the ratio of the p120/ β -catenin complex to the entire pool of p120 were relatively large as compared with the ratio of these complexes to the entire β -catenin pool. It remains unknown, however, whether all of these complexes were associated with E-cadherin. The coprecipitation of β -catenin and plakoglobin, found in the present study, appears to be inconsistent with the observation that their associations with cadherin are mutually exclusive. But, the present finding concerns a relatively minor population of these two proteins.

When 3T3 cells were transfected with E-cadherin cDNA, the level of β -catenin was upregulated. Thus it can be postulated that association with E-cadherin retards the turnover of β -catenin. Similar cadherin-dependent upregulation of catenin expression has been observed for α -catenin (Nagafuchi et al., 1991) and β -catenin (Nagafuchi et al., 1994; Nakagawa, S., and M. Takeichi, unpublished results) in other systems. In contrast, p120 expression was not affected by E-cadherin transfection, perhaps because a large amount of p120 exists without association with E-cadherin.

The most important question is what role p120 plays in cadherin function and cell adhesion. We found that the p120/E-cadherin complex contained α -catenin, a crucial component for cadherin function (Hirano et al., 1992; Shimoyama et al., 1992; Nagafuchi et al., 1994). Whether or not this α -catenin is associated directly with p120 remains to be investigated; it is possible that the coprecipitation of α -catenin with p120 was mediated by β -catenin and/or plakoglobin. p120 colocalized with E-cadherin at cell-cell contact sites, and a portion of each protein was resistant to detergent extraction, suggesting that p120 is associated with E-cadherin molecules anchored at the cytoskeleton. These observations suggest that p120 is involved in cell-cell adhesion together with E-cadherin. Considering the unique association pattern of p120 and E-cadherin and the relatively small amount of p120 in the complex, the role of p120 in the cadherin adhesion system may not be identical to that of β -catenin or plakoglobin. Perhaps p120 modulates the function of the E-cadherin/ β -catenin or E-cadherin/plakoglobin complexes by additionally binding to them, and playing a signaling role as discussed below.

It was found that p120 as well as β -catenin and plakoglobin are sensitive in tyrosine phosphorylation. Recently, we reported that treatment of various carcinoma cells, including HT29 cells, with HGF/SF or EGF for 24 h induces or enhances tyrosine phosphorylation of β -catenin, plakoglobin, and an unidentified 115-kD protein (Shibamoto et al., 1994). This 115-kD protein is very likely p120. In the present study, we found that p120 was constitutively tyrosine phosphorylated in HT29 cells. This phosphorylation was temporarily suppressed and subsequently increased by the addition of HGF/SF. The same change in tyrosine phosphorylation was observed in the 115-kD protein associated with E-cadherin. In our previous work (Shibamoto et al., 1994), we reported an increase in tyrosine phosphorylation of the 115-kD protein as early as 5 min after HGF/SF treatment. There was

a tendency for the increase in tyrosine phosphorylation to be followed by a decrease, as observed in the present work. The reproducibility of the increase in tyrosine phosphorylation remains to be tested by further experiments since the data fluctuate at earlier incubation periods with the growth factor. Although the level of tyrosine phosphorylation of p120 changed during incubation with HGF/SF, the amount of p120 associated with E-cadherin was constant, implying that tyrosine phosphorylation of p120 plays a role in interactions with molecules other than the cadherins.

It has been reported that some growth factors induce tyrosine phosphorylation on p120 within 5 min in quiescent NIH3T3 cells (Downing and Reynolds, 1991; Kanner et al., 1991). These findings are not consistent with the present observation that tyrosine phosphorylation of p120 is suppressed in response to HGF/SF. However, this discrepancy can be ascribed to differences in the cell lines used, e.g., normal vs. carcinoma lines. In HT29 cells, several Src family kinases are upregulated (Park et al., 1993), which might cause a constitutive tyrosine phosphorylation of p120. Through unknown mechanisms, the cell may respond to growth factors by either increasing or decreasing tyrosine phosphorylation of p120. In this regard, it should be noted that a protein tyrosine phosphatase activity is associated with the HGF/SF receptor, and its phosphatase activity is correlated with activation of the receptor (Villa-Moruzzi et al., 1993). Moreover, a phosphotyrosine phosphatase containing Src homology 2 domains was found to be activated in response to EGF and PDGF (Vogel et al., 1993; Feng et al., 1993). These studies, together with our results, suggest that p120, as well as β -catenin and plakoglobin, might be substrates for these phosphatases.

The response of p120, β -catenin and plakoglobin to growth factors implies they have a signaling role. In fact, studies on *Drosophila* Armadillo, a β -catenin homologue, suggest that this molecule plays a role in cell differentiation and pattern formation (Peifer et al., 1993, 1994b). A study with *Xenopus* embryos suggests that this may also be the case for vertebrates (McCrea et al., 1993). Moreover, tyrosine phosphorylation of p120 induced by Src is closely correlated with cellular transformation mediated by this kinase (Reynolds et al., 1989). We can postulate at least two different mechanisms for how these proteins play signaling roles. First, they may regulate the cadherin adhesion system, leading to a modulation of cell-cell interactions; tyrosine phosphorylation may be involved in this process. We recently found that cadherin-mediated cell-cell contacts can regulate cell growth (Watabe et al., 1994). Therefore, the regulation of this adhesion apparatus could be an important step for control of cell growth and differentiation. Second, these molecules, by themselves, may play a role in signal transduction. In *Drosophila*, cytoplasmic Armadillo proteins seem to be stabilized in response to upstream genes, and this fraction of Armadillo has been hypothesized to control downstream genes (Peifer et al., 1993; Siegfried et al., 1994; Noordermeer et al., 1994; Peifer et al., 1994b). It is noteworthy that a large amount of p120 was not associated with E-cadherin, as mentioned above, and this form of p120 could be analogous to the cytoplasmic Armadillo.

The finding that APC, a tumor suppressor gene product, interacts with β -catenin and α -catenin (Su et al., 1993; Rubinfeld et al., 1993) supports the notion that catenins are

involved in growth control. APC itself shows homology to p120, β -catenin and plakoglobin (Peifer et al., 1994a), implying that they share a common function. Interactions between these molecules or their interactions with cadherins may generate unknown but important signals to control cellular functions. p120, as a tyrosine kinase substrate, is possibly one of the key molecules involved in this complex regulatory system. The findings in this present study should aid in unraveling the molecular events taking place in such a regulatory system.

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