Identification of a New Catenin: the Tyrosine Kinase Substrate p120^{cas} Associates with E-Cadherin Complexes

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p120^{cas} is a tyrosine kinase substrate implicated in ligand-induced receptor signaling through the epidermal growth factor, platelet-derived growth factor, and colony-stimulating factor receptors and in cell transformation by Src. Here we report that p120 associates with a complex containing E-cadherin, α -catenin, β -catenin, and plakoglobin. Furthermore, p120 precisely colocalizes with E-cadherin and catenins in vivo in both normal and Src-transformed MDCK cells. Unlike β -catenin and plakoglobin, p120 has at least four isoforms which are differentially expressed in a variety of cell types, suggesting novel means of modulating cadherin activities in cells. In Src-transformed MDCK cells, p120, β -catenin, and plakoglobin were heavily phosphorylated on tyrosine, but the physical associations between these proteins were not disrupted. Association of p120 with the cadherin machinery indicates that both Src and receptor tyrosine kinases cross talk with proteins important for cadherin-mediated cell adhesion. These results also strongly suggest a role for p120 in cell adhesion.

p120 is a major Src substrate whose phosphorylation on tyrosine correlates with cell transformation (41). Unlike most Src substrates, p120 is not phosphorylated by nonmyristylated Src mutants which cannot phosphorylate critical membrane-associated substrates necessary for cell transformation. In addition, p120 is tyrosine phosphorylated in response to ligand-induced stimulation of several receptor tyrosine kinases, including those for epidermal growth factor, platelet-derived growth factor, and colony-stimulating factor 1 (6, 17). Although these observations suggest that p120 plays a role in cell transformation and ligand-induced signaling, the predicted amino acid sequence of p120 lacks motifs (e.g., SH2 and SH3 domains) that might suggest its participation in mitogenic signaling.

One striking feature of the p120 sequence is the presence of an Arm domain (36, 40) comprising 11 copies of a 42-amino-acid motif originally described for the *Drosophila* segment polarity gene product, armadillo (38, 42). The function of the Arm domain is unknown, but armadillo's vertebrate homologs, β -catenin and plakoglobin, bind to the cytoplasmic tail of E-cadherin, an interaction essential for cadherin-mediated cell-cell adhesion (29, 33). The cadherins are a family of transmembrane glycoproteins that connect cells by Ca²⁺-dependent homophilic interactions between their extracellular domains (for a review, see reference 51). Their intracellular cytoplasmic segments are thought to anchor cadherins to the actin cytoskeleton through α -catenin, β -catenin, and γ -catenin (plakoglobin). α -Catenin probably mediates the interaction with actin, because it is partially homologous to the actin-binding protein vinculin (12, 30), and its presence in complexes with cadherin correlates with the ability of these complexes to bind to DNase I actin-binding columns (33). Critical roles for β -catenin and plakoglobin in adhesion are largely inferred,

because their association with cadherin is essential for its adhesive function (29, 33).

β -Catenin associates directly with the tumor suppressor protein adenomatous polyposis coli (APC) (43, 49). Mutation of APC appears to be the first step in colon carcinogenesis (9, 19, 39), after which progression to carcinoma involves additional mutations in specific oncogenes (e.g., *ras*) and tumor suppressors (e.g., p53) (for a review, see reference 7). Interestingly, like β -catenin, plakoglobin, and p120, APC contains an Arm domain (36). The interaction between β -catenin and APC is mediated by the Arm domain of β -catenin and a region in APC consisting of three 15-amino-acid repeats (49). Arm domains may therefore be important for the protein-protein interactions that mediate cell adhesion, and they may play similar roles in proteins like p120.

Accumulating evidence suggests that in malignant cells, malfunction of cadherins contributes to the loss of cell-cell adhesion and the gain of invasiveness correlated with metastasis. In normal cells, perturbation of E-cadherin function promotes invasive behavior (1, 2, 10, 54), while the invasive potential of malignant cells is often suppressed by enforced expression of normal E-cadherin (4, 8, 54). In well-differentiated, noninvasive tumors, E-cadherin is usually highly expressed, but in invasive cells with poorly differentiated phenotypes, E-cadherin expression is lost, reduced, or heterogeneous (2, 8, 31, 44–46, 48). There are, however, exceptions to this phenomenon. In some carcinomas, the proteins mediating cadherin function (e.g., catenins) are defective. For example, in the human lung cancer cell line PC9 and the human prostate cancer cell line PC3, the α -catenin genes are deleted (28, 32, 47). When α -catenin levels are restored in PC9 cells by transfection, the cells revert to a well-differentiated epithelium (13), indicating that in these tumors, the absence of α -catenin—and presumably the consequent loss of cadherin function—probably confers metastatic ability.

Several Src family kinases have been indirectly implicated in cadherin function. Cadherins are concentrated at adherens junctions, where they regulate cell-cell recognition and tissue

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morphogenesis (51). c-Src, c-Yes, and Fyn are also enriched at adherens junctions (52), which are major sites of tyrosine-specific protein phosphorylation (55), especially during development when tissues are rapidly forming (23, 24, 50). Inhibition of tyrosine phosphatases leads to rapid increases in adherens junction-associated phosphotyrosine and deterioration of junctions. Conversely, in Src-transformed cells, adherens junctions disrupted by transformation are reformed after treatment with tyrosine kinase inhibitors (55). In MDCK cells expressing temperature-sensitive Src mutants, morphological changes are observed within minutes after a shift to the permissive temperature, suggesting that tyrosine phosphorylation directly alters cell morphology. Tyrosine phosphorylation of β -catenin is induced under these conditions (3) and might contribute to the observed morphological alterations by affecting cell adhesion. In addition, like p120, β -catenin is not phosphorylated by nonmyristylated Src mutants (11), a property shared by only a handful of Src substrates. Although the putative substrates mediating adherens junction function have not been identified, p120 and β -catenin are good candidates.

Recently, we generated antibodies specific to p120 and used them to immunolocalize p120 to sites of cell-cell contact. The similarities in structure and subcellular localization of p120 and β -catenin implied that p120 might have β -catenin-like roles and binding partners (e.g., cadherins, catenins, and APC). Here we demonstrate that p120 associates *in vivo* with cadherin-catenin complexes and precisely colocalizes with E-cadherin and catenins in normal and Src-transformed cells. p120 is therefore indistinguishable in most respects from β -catenin and plakoglobin, which have similar structures, locations, binding partners, and probably functions. We have named p120 Cas, which stands for cadherin-associated Src substrate.

MATERIALS AND METHODS

Cell culture and plasmids. NIH 3T3 (murine fibroblast) and MDCK (canine kidney epithelial) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2% L-glutamine, penicillin (100 U/ml), and streptomycin (100 μ g/ml). Clonal cell lines of Src-transformed MDCK cells were established by cotransfection with pSVneo and a Moloney long terminal repeat-based plasmid containing *v-src* (pMvsrc) (15, 20), followed by selection in G418. MDCK cells were radiolabeled for 12 h by the addition of 50 μ Ci of [³⁵S]methionine per ml to methionine-free culture medium containing dialyzed fetal calf serum. Cells were lysed, immunoprecipitated, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described below. Radiolabeled proteins were detected by fluorography with En³Hance (NEN-Dupont, Boston, Mass.).

To facilitate cloning of the p120 gene into the pRc/CMV expression vector (Invitrogen, San Diego, Calif.), an *Eco*RI site was introduced 14 nucleotides upstream of the start codon in cDNA clone 88a (40) by oligonucleotide-directed mutagenesis as described previously (41). Similarly, a *Kpn*I site was introduced 10 nucleotides downstream from the stop codon, and the *Eco*RI-*Kpn*I fragment was ligated into these sites in the pRc/CMV multiple cloning site to yield pRc/CMVp120. Carboxy-terminal epitope-tagged p120 was generated by the addition of a sequence which encoded three tandemly linked copies of the 9-amino-acid epitope for the antipeptide monoclonal antibody (MAb) 12CA5 (pRc/CMVp120^{tag}). Plasmids were transiently transfected into MDCK cells with lipofectamine (Gibco-BRL, Gaithersburg, Md.).

Immunoprecipitation and Western blotting (immunoblotting). Except where noted, cells were lysed with 0.5% Nonidet P-40 (NP-40) in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.1 mM sodium vanadate, and 0.1 trypsin inhibitor units of aprotinin and 5 μ g of leupeptin per ml (protease inhibitors from Sigma, St. Louis, Mo.). For analysis of cadherin complexes in different detergents, 0.5% NP-40 was replaced with 1% NP-40–0.25% deoxycholate or 1% digitonin. Proteins were immunoprecipitated from the lysates, separated by SDS-PAGE (21), and transferred to nitrocellulose filters by methods described previously (16, 41). Antiphosphotyrosine blots were blocked overnight at room temperature in Tris-buffered saline (TBS), pH 7.4, containing 5% bovine serum albumin. All other blots were blocked with TBS containing 3% nonfat dry milk. Filters were washed five times in TBS after each antibody incubation. The filters were incubated with the primary antibody (0.2 to 2.0 μ g/ml) overnight at 4°C in TBS–3% milk. The anti-p120 MAb 2B12 (18), the anti-Src MABs EC10 (34) and 327 (22), and the anti- β -catenin polyclonal antisera (27, 37) have been described elsewhere. The antiplakoglobin MAb 5F11 and the anti- α -catenin MAb IG5 were generated as described previously (14). The anti-p120 MAb pp120, generated against the C-terminal amino acids 790 to 911 of p120, and the antiphosphotyrosine MAB PY20 were purchased from Transduction Laboratories (Lexington, Ky.). Peroxidase-conjugated donkey anti-mouse and donkey anti-rabbit antibodies and peroxidase-conjugated protein A (Jackson ImmunoResearch Laboratories, West Grove, Pa.) were diluted 1/50,000 in blocking buffer and applied to the filters for 2 h at room temperature. The blots were developed with the enhanced chemiluminescence system (Amersham, Arlington Heights, Ill.) according to the manufacturer's instructions.

V8 peptide mapping. p120 isoforms were immunoprecipitated from NIH 3T3 cell lysates with MAB pp120, separated on 5% polyacrylamide gels containing 0.1% SDS, and processed for peptide mapping with *Staphylococcus* V8 protease as described previously (5). The proteins were digested in the stacking gel and then separated on a 13% polyacrylamide denaturing gel, transferred to nitrocellulose, and probed with an antibody cocktail containing MAB pp120 (0.25 μ g/ml), MAB 2B12 (1 μ g/ml), polyclonal antibody (PAb) F1 (0.25 μ g/ml), and PAb F3 (1 μ g/ml).

Immunofluorescence. Cells were grown on glass coverslips, rinsed briefly in phosphate-buffered saline (PBS), pH 7.4, and fixed for 15 min in cold 4% paraformaldehyde. The cells were then permeabilized by a 10-min treatment with 0.2% Triton X-100 in PBS. The titers of the primary antibodies were determined, and the antibodies were applied at 0.5 to 10 μ g/ml in PBS for 30 min at room temperature. The coverslips were washed, and secondary antibodies (diluted 200-fold) were applied for 30 min at room temperature. The secondary antibodies were donkey anti-mouse and donkey anti-rabbit immunoglobulin G coupled to fluorescein isothiocyanate or lissamine rhodamine (Jackson ImmunoResearch Laboratories).

RESULTS

Cell-type-specific expression of Cas isoforms. The new antibodies to Cas recognized two additional proteins on Western blots that were not seen with our original MAB 2B12, suggesting that Cas might have isoforms or close family members. To characterize these proteins, we immunoprecipitated them with a panel of five specific anti-Cas antibodies for analysis by Western blotting (Fig. 1). In lysates from murine NIH 3T3 fibroblasts and canine MDCK epithelial cells (Fig.

FIG. 1. Isoforms identified by different antibodies to p120^{cas}. (A) Immunoprecipitates from NIH 3T3 fibroblasts and MDCK epithelial cells obtained with the indicated antibodies were separated by SDS-PAGE and Western blotted with MAb pp120. The four forms of p120^{cas} found in NIH 3T3 cells are indicated on the left. P-A/R M, protein A-Sepharose beads with rabbit anti-mouse immunoglobulin G; P-A, protein A-Sepharose beads alone. (B) Schematic of p120^{cas} illustrating the armadillo repeats (shaded boxes), the approximate locations of epitopes for Mabs 2B12 and pp120, and the three p120^{cas} fragments used to generate and affinity purify Pabs F1, F2, and F3. (C) MDCK cells were double immunolabeled with Pab F1 (a) and MAb pp120 (b), demonstrating identical staining patterns. No staining was obtained with identical concentrations of protein A-purified preimmune rabbit immunoglobulin G (c).

1A), the original anti-Cas MAb 2B12 recognized two proteins migrating at ~115 and 112 kDa, which we have termed Cas1 isoforms A and B (Cas1A and Cas1B), respectively. The new MAb (pp120) and each of the new affinity-purified Pabs (F1, F2, and F3) recognized two additional proteins migrating between 90 and 105 kDa, which we have termed Cas2A and Cas2B. NIH 3T3 cells expressed mainly Cas1 isoforms, whereas MDCK cells preferentially expressed Cas2 isoforms. Figure 1B illustrates schematically the p120^{cas} epitopes for the two Mabs (2B12 and pp120) and the p120^{cas} fragments recognized by the three affinity-purified rabbit Pabs (F1, F2, and F3). In pilot experiments, Pab F1 displayed the highest titer for immunofluorescent localization of Cas. Figure 1C demonstrates that this antibody can be used interchangeably with MAb pp120; both antibodies localized Cas to cell-cell boundaries, and they displayed identical staining patterns (Fig. 1C, panels a and b, respectively), whereas staining was not detected with preimmune antibodies (Fig. 1C, panel c).

To determine whether the four species of Cas were highly related isoforms or more distantly related family members, the four bands immunoprecipitated from NIH 3T3 cell lysates by MAb pp120 were analyzed by V8 protease mapping (Fig. 2). The peptides were detected on Western blots by a pool of four different antibodies specific for Cas. The peptide maps from all four forms of Cas were nearly identical, indicating that the proteins are isoforms.

A subset of Cas1B and Cas2B peptides migrated faster than their Cas1A and Cas2A counterparts (Fig. 2A), suggesting that these fragments might contain alternative splicing inserts. During the initial characterization of p120^{cas}, the murine cDNA encoding Cas1B was isolated (40). In Northern (RNA) blot analysis, this cDNA detected a single diffuse band of ~6 kb. However, restriction analysis of additional cDNA clones revealed two classes of cDNAs which differed by a 63-nucle-

otide insertion at codon 874 (Fig. 2B). Thus, the higher-molecular-weight form of Cas observed in murine fibroblasts, now termed Cas1A, is an alternatively spliced variant of Cas1B containing a 21-amino-acid insert near the C-terminal end. We have yet to clone and sequence the Cas2A and -2B isoforms to pinpoint the specific differences between them.

Since there are differences in electrophoretic mobility between the murine NIH 3T3 and the canine MDCK isoforms (Fig. 1A), it will not be possible to directly compare the Cas isoforms of different species until they are cloned and sequenced. In the meantime, we propose that Cas isoforms of different species be referred to as two groups on the basis of their reactivities with MAb 2B12: Cas1 isoforms, which contain the N-terminal sequence defined by the MAb 2B12 epitope, and Cas2 isoforms, which lack this sequence. In NIH 3T3 cells, the Cas1 proteins can be subdivided into isoforms that differ by the presence (Cas1A) or absence (Cas1B) of the 21-amino-acid C-terminal insertion shown in Fig. 2B. On the basis of the peptide maps, Cas2A and Cas2B are likely to differ by the same sequence. Since we detect four main isoforms for most species (data not shown), it is likely that this nomenclature will be widely applicable.

Cas interacts with cadherin-associated proteins. In our preliminary attempts to find Cas binding partners, MDCK cells were metabolically labeled with [³⁵S]methionine and cell lysates were immunoprecipitated with antibodies to Cas, E-cadherin, or the catenins (Fig. 3). However, the Cas2 isoforms (Fig. 3, lane 2) migrated with the same electrophoretic mobility as the previously identified E-cadherin-binding proteins, α - and β -catenin (lanes 3 through 6), and were therefore indistinguishable by fluorography.

Subsequently, we tagged Cas1A with the influenza virus peptide sequence recognized by MAb 12CA5. This antibody does not react with untagged Cas or any of the cadherin-

FIG. 2. Characterization of p120^{cas} isoforms and alternative splicing. (A) Peptide maps were generated from the indicated Cas isoforms with increasing amounts of V8 protease. The peptides were separated on a 13% polyacrylamide gel, transferred to nitrocellulose, and detected by Western blotting with a cocktail of anti-p120 antibodies (as described in Materials and Methods). The maps are nearly identical, except for a few shifted bands (indicated by thick horizontal bars) which are likely to differ by the alternatively spliced sequence shown in panel B. (B) Nucleotide and amino acid sequences of the 63-nucleotide insertion at codon 874 in *cas1B*.

associated proteins and was used as a negative control for the other experiments. Indeed, neither Cas1A nor β -catenin was detected in immunoprecipitates made with MAb 12CA5 when cells were transfected with wild-type (untagged) Cas1A (Fig. 4, lanes 1 and 3, respectively). However, when the vector encoding epitope-tagged Cas1A was transiently transfected into MDCK cells (Fig. 4), MAb 12CA5 coprecipitated the tagged Cas1A (lane 2) and β -catenin (lane 4). Thus, β -catenin was specifically associated with the Cas1A protein.

Next, the complexes were characterized in more detail by using a variety of detergent lysis conditions (Fig. 5). With antibodies to Cas and to the cadherin-associated proteins plakoglobin and β -catenin, immunoprecipitates from MDCK cells were prepared in lysis buffers containing either 1% NP-40–0.25% deoxycholate (Fig. 5A), 0.5% NP-40 (Fig. 5B), or 1% digitonin (Fig. 5C). When gel separations of these immunoprecipitates were blotted with the antibodies listed at the bottom of Fig. 5, we detected stable protein-protein complexes containing Cas1 or Cas2 isoforms, β -catenin, plakoglobin, and E-cadherin.

The compositions of these complexes varied depending on the detergent used but were not due to nonspecific interac-

tions, since an irrelevant antibody (Fig. 5, lanes 1, 6, 11, and 16) did not bring down any of the proteins nor did any of the protein-specific antibodies cross-react with other proteins (data not shown).

Complexes containing Cas (Fig. 5, lanes 2 through 5) were most apparent in the preparations containing either 0.5% NP-40 (Fig. 5B) or 1% digitonin (Fig. 5C). Under these conditions, β -catenin and E-cadherin were detectable not only in Cas immunoprecipitates made from the pan-specific Cas antibody, MAb pp120, but also in those made with the Cas1-specific MAb 2B12 (Fig. 5, lanes 7 and 17). Plakoglobin was detectable in immunoprecipitates made with MAb pp120 but not MAb 2B12. The absence of plakoglobin from MAb 2B12 immunoprecipitates probably reflects the low abundance of the Cas1 isoforms in MDCK cells combined with the relatively low affinity of plakoglobin in cadherin-associated complexes.

Interestingly, in lysates made with 1% digitonin (Fig. 5C), two distinct complexes which were almost mutually exclusive for either β -catenin or plakoglobin were detected. The anti-E-cadherin blot in Fig. 5C shows that equivalent amounts of E-cadherin were present in the 1% digitonin immunoprecipi-

FIG. 3. Comigration of MDCK Cas2 isoforms with α - and β -catenins. MDCK cells were radiolabeled with [35 S]methionine, lysed in buffer containing 1% NP-40 and 0.25% deoxycholate, and analyzed by immunoprecipitation with the indicated antibodies. The Cas2 isoforms of p120 cannot be distinguished from α - and β -catenins (α -cat and β -cat, respectively) by fluorography. E-cad, E-cadherin; α -cat, α -catenin; β -cat, β -catenin; plak, plakoglobin.

tates whether they were made with antibodies to Cas, plakoglobin, or β -catenin (lanes 18, 19, and 20, respectively). However, despite the fact that β -catenin was efficiently immunoprecipitated in 1% digitonin (Fig. 5C, lane 10), barely detectable levels of plakoglobin immunoprecipitated with it, compared with the amount that coprecipitated in the 0.5% NP-40 preparation (compare lanes 15 in Fig. 5C and B); at the same time, the amounts of plakoglobin present in the Cas immunoprecipitates in each case were comparable (compare lanes 13 in Fig. 5C and B). Likewise, in 1% digitonin, very little β -catenin coprecipitated with plakoglobin (compare lanes 9 in Fig. 5C and B), despite an abundance of plakoglobin in the immunoprecipitate (Fig. 5C, lane 14) and the fact that the amount of β -catenin coming down with Cas remained about the same in 1% digitonin as was detected in 0.5% NP-40 (lanes 8 in Fig. 5C and B, respectively). Thus, the complexes obtained from NP-40 lysates contain both plakoglobin and β -catenin, whereas digitonin apparently disrupts an as-yet-unknown protein-protein interaction, revealing two distinct complexes that are almost mutually exclusive for β -catenin and plakoglobin.

Effects of Src transformation and tyrosine phosphorylation on complexes. Lysates from normal and v-Src-transformed MDCK cells were immunoprecipitated with a panel of antibodies as indicated in Fig. 6. These immunoprecipitates were Western blotted with antibodies to phosphotyrosine (Fig. 6A), Cas (Fig. 6B), cadherin and its associated proteins (Fig. 6C through F), and Src (Fig. 6G).

FIG. 4. Specific coprecipitation of β -catenin with p120^{cas1A}. p120^{cas1A} was epitope tagged at its C-terminal end with a 9-amino-acid peptide from influenza virus which is recognized specifically by MAb 12CA5, the MAb normally used as our negative control. Constructs encoding untagged (lanes 1 and 3) and tagged (lanes 2 and 4) p120^{cas1A} were transiently transfected into MDCK cells. Cell lysates were immunoprecipitated with MAb 12CA5 and Western blotted with MAb 12CA5 or anti- β -catenin (anti- β -cat). Wild-type p120^{cas} was not precipitated by MAb 12CA5, nor was β -catenin, whereas tagged p120^{cas1A} was specifically precipitated by MAb 12CA5 and β -catenin was coprecipitated.

Except for Src, none of the proteins from normal MDCK cells was phosphorylated on tyrosine (Fig. 6A, lanes 1 through 8).

In Src-transformed cells, both Cas1 and Cas2 isoforms were heavily phosphorylated on tyrosine (Fig. 6A, lanes 9 and 10), as was plakoglobin (Fig. 6A, lanes 10 through 14). This is the first observation of tyrosine phosphorylation of plakoglobin in Src-transformed cells. β -Catenin also appeared to be phosphorylated in the transformed cells (Fig. 6A, lanes 11 through 14), but since it comigrated with Cas2B, we could not distinguish the two. Only minimal, if any, tyrosine phosphorylation was detected for E-cadherin and α -catenin.

Tyrosine phosphorylation of plakoglobin did not prohibit its participation in complexes, as evidenced by the presence of tyrosine-phosphorylated plakoglobin in Cas, α -catenin, β -catenin, and E-cadherin immunoprecipitates (Fig. 6A, lanes 10 through 12 and 14). In a separate experiment, we compared the extent of tyrosine phosphorylation of plakoglobin that was directly immunoprecipitated with an antiplakoglobin antibody (MAb ISF11) with that obtained from complexes isolated with an antibody to E-cadherin (MAb rr1) (data not shown). When the amount of plakoglobin obtained with these two antibodies was normalized and then the plakoglobin was probed by Western blotting with antibodies to phosphotyrosine, the two

FIG. 5. Effects of detergents on the coprecipitation of Cas with cadherin-associated proteins. With the antibodies indicated across the top, cadherin-associated proteins were immunoprecipitated from MDCK cell lysates made with buffers containing 1% NP-40 and 0.25% deoxycholate (DOC) (A), 0.5% NP-40 (B), or 1% digitonin (C). Coprecipitating proteins were detected by Western blotting with antibodies to p120 (MAb pp120, lanes 1 through 5), β -catenin (lanes 6 through 10), plakoglobin (MAb I5F11, lanes 11 through 15), and E-cadherin (MAb rr1, lanes 16 through 20). Plak, antiplakoglobin; β -cat, anti- β -catenin.

populations of plakoglobin were found to contain identical levels of phosphotyrosine.

In the β -catenin, plakoglobin, and E-cadherin immunoprecipitates (Fig. 6A, lanes 12 through 14), two other tyrosine-phosphorylated bands were reproducibly detected; these may be Cas isoforms that migrate more slowly than normal because of phosphorylation. Alternatively, they may be unrelated to Cas, in which case tyrosine-phosphorylated Cas may be excluded from cadherin complexes.

Figure 6B through F show quantitatively the complexes obtained from the normal and Src-transformed MDCK cells. No significant differences were observed, indicating that the compositions of the complexes were not grossly altered by transformation or tyrosine phosphorylation.

Colocalization of p120 and E-cadherin-associated proteins. Double-immunofluorescent-labeling experiments with MDCK cells and antibodies to Cas, E-cadherin, α -catenin, β -catenin, and plakoglobin demonstrated striking colocalization of Cas with all of these proteins (Fig. 7). Aside from a bright thin band of desmosomal staining noticed with antiplakoglobin antibodies, there were no noticeable differences in costaining in any plane of focus. The precise colocalization of the various proteins persisted even in v-Src-transformed cells, in which much of the sharp junctional staining of normal cells was replaced by diffuse staining.

c-Src was detected in nontransformed cells at cell-cell junctions, and this staining coincided with that of p120^{Cas} (Fig. 7A, panels i and j), although unlike the staining of cadherin-associated proteins, c-Src staining did not only coincide with staining for p120^{Cas}. Additional c-Src staining was diffusely distributed at the cell surface, and some cells displayed staining of structures that resembled the nuclear organizing region. In v-Src-transformed cells, some of the Src staining occurred in focal adhesions, which were identified by colocalization with talin (data not shown). p120^{Cas} did not colocalize with Src in focal adhesions. Although much of the v-Src staining was diffuse, a significant amount of staining colocalized with that of p120^{Cas} (Fig. 7B, panels s and t). This was most obvious in the

punctate structures observed in many of these cells (Fig. 7B, panel t).

DISCUSSION

The work presented here links the Src and receptor tyrosine kinase substrate p120^{Cas} to proteins regulating cadherin-mediated cell adhesion. We found that specific antibodies to α -catenin, β -catenin, plakoglobin, and E-cadherin immunoprecipitated complexes containing isoforms of Cas. Likewise, two different MAbs to Cas specifically brought down complexes containing the above-mentioned cadherin-associated proteins and E-cadherin. These experiments indicate that Cas1 and Cas2 isoforms associate, directly or indirectly, with E-cadherin. (We suspect that Cas isoforms have been present in cadherin immunoprecipitates from other laboratories but were masked by comigrating α - and β -catenins.) These binding data, together with our colocalization of Cas isoforms with cadherin-associated proteins and the structural similarity of Cas to β -catenin and plakoglobin (36, 40), indicate that Cas is a member of the group of cadherin-associated proteins called catenins.

Interestingly, immunoprecipitations in 1% digitonin yielded two apparently distinct complexes, one containing Cas, β -catenin, and E-cadherin and the other containing Cas, plakoglobin, and E-cadherin. The nearly complete separation of these complexes was striking; β -catenin was almost nonexistent in plakoglobin immunoprecipitates and vice versa, whereas E-cadherin associated equivalently with Cas, β -catenin, and plakoglobin. The binding of β -catenin and that of plakoglobin to a single cadherin molecule may therefore be mutually exclusive events. In 0.5% NP-40, β -catenin and plakoglobin were present in the same complex, suggesting the possibility that the two distinct complexes (found in the presence of digitonin) form a higher-order complex in vivo that is disrupted in digitonin buffer but not in 0.5% NP-40 buffer. In future work, mapping the direct contacts between these proteins and determining the stoichiometry of the proteins in the complexes

FIG. 6. Comparison of cadherin complexes from normal and Src-transformed cells and tyrosine phosphorylation of p120^{cas}, β-catenin, and plakoglobin. Normal (left) or Src-transformed (right) MDCK cells were lysed in 0.5% NP-40, and cadherin-associated proteins were immunoprecipitated with the antibodies indicated across the top. Immunoprecipitated proteins were detected by Western blotting with the antibodies indicated on the left. The locations of E-cadherin (E-cad), α-catenin (α), β-catenin (β), plakoglobin (plak), and c-Src and/or v-Src (Src) are indicated on the right. anti-pTyr, antiphosphotyrosine.

will be important for elucidating the mechanism of cadherin-mediated cell adhesion.

The detection of Cas isoforms in the various complexes described above raises the possibility that cadherin function

might be regulated by selective expression of the Cas isoforms in different cell types. No isoforms of β-catenin and plakoglobin have been reported, although, using antibodies to plakoglobin, we consistently detected a second protein migrating

FIG. 7. Colocalization of p120^{cas} with E-cadherin-associated proteins and Src in normal and Src-transformed MDCK cells. Normal (A) and v-Src-transformed (B) MDCK cells were double labeled by immunofluorescence with rabbit anti-Cas PAb F1 (a, c, e, g, i, k, m, o, q, and s) and murine MAbs to E-cadherin (MAb rr1; b and l), α -catenin (MAb IG5; d and n), β -catenin (MAb 10C4; f and p), plakoglobin (MAb I5F11; h and r), and Src (MAb 327; j and t). p120^{cas} and cadherins colocalized even after Src transformation, which induced cell scattering and more diffuse staining patterns.

slightly faster than plakoglobin. Two neuron-specific isoforms of α -catenin have been identified, but these are relatively restricted in their tissue distribution (13, 53). Thus, Cas is unique among the catenins in that four isoforms are expressed simultaneously in most adherent cell types (Fig. 1 and unpublished data). In NIH 3T3 cells, which express N-cadherin and P-cadherin but not E-cadherin, Cas1 isoforms were most strongly expressed, whereas in MDCK cells, which express only E-cadherin, Cas2 isoforms were predominant. In future work it will be important to determine whether specific Cas isoforms interact with different cadherins or whether their expression is associated with specific events like the epithelium-to-mesenchyme transitions that occur during development, wound healing, and metastasis.

Although multiple lines of evidence indicate that cadherins do not function efficiently in Src-transformed cells (3, 11, 25), our data did not reflect any changes in the physical interactions of cadherin-associated proteins under conditions in which they were phosphorylated on tyrosine. The levels of each cadherin-associated protein in complexes from normal and transformed cells were very similar, indicating that no single member of the complex is selectively excluded as a result of transformation. Although on antiphosphotyrosine Western blots some of the bands could not be distinguished from one another because of comigration (e.g., Cas2 isoforms comigrated with α - and β -catenin), it was clear in the case of plakoglobin that tyrosine phosphorylation did not preclude its participation in the complex. Similarly, Hamaguchi et al. (11) found apparently

normal N-cadherin complexes in Src-transformed fibroblasts, and these complexes contained tyrosine-phosphorylated catenins. Further experiments, perhaps mutational analyses of the sites of tyrosine phosphorylation, will be necessary to determine whether the phosphorylation of catenins is relevant to cell transformation.

One hypothesis consistent with our data is that Src-induced phosphorylation of cadherin-associated proteins leads to dissociation of intact cadherin complexes from the underlying actin cytoskeleton and lateral diffusion of untethered complexes in the cell membrane. In addition to the diffusely localized staining of cadherin-associated proteins, we also observed significant colocalization of v-Src and Cas in punctate structures in v-Src-transformed cells. It is possible that phosphorylation of cadherin complexes by Src leads to internalization and degradation of components of the cell-cell adhesive junctions, in which case the punctate structures might be vesicles containing both v-Src- and cadherin-associated proteins. Although in theory, tyrosine-phosphorylated Cas could bind to the SH2 domain of Src, we found no biochemical evidence to support a significant physical association between Cas and Src.

Association of Cas with the cadherin machinery implies that Cas has an important regulatory role in cadherin function. The original role of Cas as a Src and receptor tyrosine kinase substrate implies a direct link between receptor tyrosine kinase signaling pathways and the regulation of cell adhesion. The recent additions of the tumor suppressor APC (43, 49) and these multiple Cas isoforms to the group of proteins interacting with catenins or E-cadherin complicates the previous models for the regulation of cadherin function (26, 35, 51). Future work will be directed toward understanding the role of Cas and tyrosine phosphorylation in cadherin-mediated cell-cell adhesion.

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