Abstract. p120<sup>thn</sup> binds to the cytoplasmic domain of cadherins but its role is poorly understood. Colo 205 cells grow as dispersed cells despite their normal expression of E-cadherin and catenins. However, in these cells we can induce typical E-cadherin–dependent aggregation by treatment with staurosporine or trypsin. These treatments concomitantly induce an electrophoretic mobility shift of p120<sup>thn</sup> to a faster position. To investigate whether p120<sup>thn</sup> plays a role in this cadherin reactivation process, we transfected Colo 205 cells with a series of p120<sup>thn</sup> deletion constructs. Notably, expression of NH<sub>2</sub>-terminally deleted p120<sup>thn</sup> induced aggregation. Similar effects were observed when these constructs were introduced into HT-29 cells. When a mutant N-cadherin lacking the p120<sup>thn</sup>-binding site was introduced into Colo 205 cells, this molecule also induced cell aggregation, indicating that cadherins can function normally if they do not bind to p120<sup>thn</sup>. These findings suggest that in Colo 205 cells, a signaling mechanism exists to modify a biochemical state of p120<sup>thn</sup> and the modified p120<sup>thn</sup> blocks the cadherin system. The NH<sub>2</sub> terminus–deleted p120<sup>thn</sup> appears to compete with the endogenous p120<sup>thn</sup> to abolish the adhesion-blocking action.

Key words: E-cadherin • catenin • colon carcinoma • Colo 205 • p120<sup>thn</sup>

Cell–cell adhesion is a dynamic process, especially during morphogenetic cell rearrangement such as intercalation and delamination of cells, and in many pathological phenomena including tumor invasion. To clarify the mechanisms of how cell–cell adhesion is regulated is an important issue for understanding such biological phenomena. Cadherin-mediated adhesion is thought to be controlled by catenins which are associated with the cytoplasmic domain of cadherin. A region in the COOH half of the cadherin cytoplasmic domain directly binds β-catenin, which in turn associates with α-catenin (Barth et al., 1997). α-Catenin interacts with the actin-based cytoskeleton directly or indirectly, involving other cytoskeletal proteins such as α-actinin and vinculin (Knudsen et al., 1995; Rimm et al., 1995; Watabe-Uchida et al., 1998). These molecular interactions are essential for this system to exert its full adhesion activity (Hirano et al., 1992; Watabe et al., 1994; Ozawa and Klem, 1998a; Watabe-Uchida et al., 1998), although homophilic interactions between the cadherin extracellular domains per se can be achieved without catenins (Brieher et al., 1996; Y ap et al., 1997; Chitaev and Troyanovsky, 1998). It is thought that cadherin function may be regulated by biochemical modifications of the catenins. Indeed, phosphorylation of catenins, in particular β-catenin, has been implicated in the instability of cell–cell adhesion (e.g., Matsuyoshi et al., 1992; Hamaguchi et al., 1993; Serres et al., 1997; Takaishi et al., 1997; Balsamo et al., 1998).

One such catenin is p120<sup>thn</sup>, a member of the Armadillo/β-catenin gene family (Peifer et al., 1994). The p120<sup>thn</sup> protein was originally identified as a target for p60<sup>src</sup> kinase (Reynolds et al., 1989, 1992) and later found to be a protein associated with the cytoplasmic domain of cadherins (Reynolds et al., 1994; Daniel and Reynolds, 1995; Shibamoto et al., 1995; Staddon et al., 1995). Other proteins related to p120<sup>thn</sup>, constituting a subfamily of Armadillo/β-catenin, have also been identified (H eid et al., 1994; Hatzfeld and Nachtsheim, 1996; Mertens et al., 1996; Paffenholz and Franke, 1997; Sirotkin et al., 1997). Recent studies show that p120<sup>thn</sup> binds to the juxtamembrane portion of the cadherin cytoplasmic domain, which is different from the region to which β-catenin binds (Finnemann et al., 1997; Lampugnani et al., 1997; Y ap et al., 1998). In contrast to the well-known function of β-catenin, that of...
p120<sup>ctn</sup> remains largely unknown. However, some biological effects of its ectopic expression have been reported, e.g., overexpression of p120<sup>ctn</sup> induces extensive dendrite-like processes in fibroblasts (Reynolds et al., 1996) and perturbs gastrulation in X enopus laevis embryos (Geis et al., 1998; Paulson et al., 1999). In addition, a recent report shows that overexpression of β-catenin in MDCK cells, a protein related to p120<sup>ctn</sup>, alters their morphology and motility (Lu et al., 1999).

In the present work, we studied a unique aggregation property of colon carcinoma Colo 205 cells (Semple et al., 1978). They grow as dispersed cells not forming compact aggregates, despite the expression of all general components of the E-cadherin–catenin complex. We found that typical E-cadherin–dependent aggregation could be induced by treatment with staurosporine, a kinase inhibitor, or with low concentrations of trypsin. Correlating with this adhesive change, the electrophoretic mobility of p120<sup>ctn</sup> was altered. Furthermore, when NH<sub>2</sub> terminus-deleted p120<sup>ctn</sup> molecules were introduced into Colo 205 cells, these constructs induced an E-cadherin–dependent compact aggregate formation, similar to effects induced by staurosporine and trypsin. Together with other findings, our results suggest that p120<sup>ctn</sup> can function as an inhibitory regulator in the cadherin adhesion system.

### Materials and Methods

#### Antibodies and Other Reagents

Mouse mAb b HECD-1 (Shimoyama et al., 1989) and SH E78-7 to human E-cadherin (Takara Shuzo Co., Ltd.), rat mAb b NCD-2 to chicken N-cadherin (Nakagawa and Takeuchi, 1998), mouse mAb a to p120<sup>ctn</sup> (Transduction Laboratories), mouse mAb b M2 to FLAG (F-3165; Sigma Chemical Co.), rabbit polyclonal antiserum to FLAG (SC-807; Santa Cruz Biotechnology, Inc.), mouse mAb to β-catenin (Transduction Laboratories), rat mAb b 18 to β-catenin (Watatue et al., 1994), mouse mAb SH 10 to β-catenin (Johnson et al., 1993), and rabbit polyclonal antiserum to β-catenin (Shibamoto et al., 1995) were used. Anti-MU1C antibody MY.1E12 (Yamamoto et al., 1996) was a gift from T. Irimura (University of Tokyo, Tokyo, Japan).

Ribonuclease inhibitors were used for detection of primary antibodies as follows: goat Cy-3-labeled species-specific antibody to mouse IgG (AP-124C; Chemicon International, Inc.), donkey biotinylated species-specific antibody to rabbit IgG (RPN 1004; Nycomed-Amersham), FITC-labeled streptavidin (RPN 1233; Nycomed-Amersham), sheep HRP-linked species-specific antibody to mouse IgG (NA 9310; Nycomed-Amersham) and rat IgG (NA 932; Nycomed-Amersham), goat HRP-linked antibody to rabbit IgG (NA 934; Nycomed-Amersham), and Sepharose 4B-linked goat antibody to mouse IgG (62-6541; Zymed Labs, Inc.).

The following protein kinase inhibitors were used: staurosporine (#19-123; Upstate Biotechnology), calphostin C (C-159; Research Biochemicals Inc.), tyrphostin E1 (215; Biomol), genistein (A103-9; Research Biochemicals International), and herbimycin A (OP-12, K yowa Medex Co., Ltd.). UCN-02 (Takahashi et al., 1990) was a kind gift of T. Tamaoki (Kyowa Medex Co., Ltd.). O-sialoglycoprotein endopeptidase was purchased from Cedarlane Labs, Ltd.

**Cells and cDNA Transfection**

Human colon carcinoma cell lines Colo 205 (Semple et al., 1978) and HT-29 (Fogh and Trempe, 1975), and MDCK cells (Gash et al., 1996) were used. These cells were cultured in a 1:1 mixture of DMEM and Ham’s F12 supplemented with 10% FCS (DH10). For transfer of Colo 205 cells, an aliquot of the suspended cell culture was moved to new dishes with fresh DH10 medium. When necessary, they were trypsinized as described below. For HT-29 and MDCK cells, they were rinsed with 1 mM EDTA in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free saline, then treated with 0.05% crude trypsin (#0352-13; Difco Laboratories) and 1 mM EDTA in the same solution for 5 min at room temperature, and finally suspended in DH10.

Colo 205 cells were transfected by electroporation or by use of adenoviral expression vectors. For electroporation, trypsinized cells (4 × 10<sup>6</sup>) were suspended in 200 ml of Hepes-buffered (pH 7.4) saline with 1 mM Ca<sup>2+</sup> and 1 mM Mg<sup>2+</sup> (HBS). 20 μg of an expression vector was added to the suspension, which was electriified at 1,160 μF at 250 V. For adenovirus-mediated transfection, 10<sup>6</sup> cells were suspended in 500 μl of DH10 with 5 × 10<sup>6</sup> plaque-forming units of adenovirus, and incubated for 4 h. Cells were washed twice with DH10, and after 48 h samples were collected. MDCK cells were transfected by electroporation under the same conditions as for Colo 205 cells, except that 1,060 μF at 200 V was used. HT-29 cells were transfected with adenoviral vectors only.

**Cell Aggregation and Trypsin Treatment**

Colo 205 cells were washed twice with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free Hepes-buffered saline supplemented with 1 mM EDTA, and completely dissociated into single cells by pipetting. Then, 2 × 10<sup>6</sup> cells were resuspended in 1 ml of DH10 with or without 1 μg/ml of SHE 78-7, a blocking antibody to human E-cadherin, and placed in a Nunc 6-well plate (#352795). Cells were incubated for 3 h at 37°C on a gyratory shaker (K86320; Mersoyl) at 80 rpm.

For trypsin treatment to induce cell aggregation, cells were rinsed twice with DH (DH10 without FCS), and incubated in DH with crystalline trypsin (T-T253; Sigma Chemical Co.) of various concentrations at 37°C. For biochemical analysis, the incubation was generally stopped at 30 min, and the cells were rinsed twice with HBS containing 0.1% trypsin inhibitor (T-6522; Sigma Chemical Co.) and subjected to further analysis. 0.01% trypsin in the presence (TC) or absence (TE) of 1 mM Ca<sup>2+</sup> treatments were performed as described previously (Takeichi, 1997).

**cDNA Construction**

Using mouse p120<sup>ctn</sup> cDNA (GenBank accession number Z17804) as a template, we generated FLAG-tagged p120<sup>ctn</sup> (FLI) and other constructs by PCR. The following primers were used: primer N, 5'-GA AT TCT ATG-3'; primer A, 5'-GA AT TT CAT GTG-3'; primer I, 5'-GA AT TT CAT GTG AG TGG-3'; primer C, 5'-GA AT TT CAT GTG AAG CAA-3'; primer CR, 5'-CT GC GTCA TGT GCG TAC GCT GAC CAG GGG-3'; primer D R f, in which aa 641–819 were deleted, oligonucleotides corresponding to aa 346–640 were synthesized by PCR, and then inserted into the internal PstI sites of FLI or AN346. The obtained fragments were subcloned into the pc-PA expression vector (Niwa et al., 1991) and pA Δv-CA-pA ade-

1. Abbreviations used in this paper: aa, amino acid; FLI, full-length FLAG-tagged p120<sup>ctn</sup>.

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**Construction of Recombinant Adenovirus**

Recombinant adenovirus Adv-CA-lacZ expressing β-galactosidase with the CAG promoter was a gift from K. Moriyoshi (Kyoto University, Kyoto, Japan). Construction of recombinant viruses was performed according to methods described previously (Moriyoshi et al., 1996). In brief, HEK 293 cells (ATCC CRL 1573) cultured with D/H 10 in 6-well plates (Iwaki Co.) were cotransfected with viral genome fragments (0.2 μg) and linearized adenoviral shuttle vector plasmids (1 μg) by use of Lipofectamine™ (#18324-012; Life Technologies, Inc.). The next day, the cells were divided into collagen-coated 24-well plates (Iwaki Co.). 10 d later, wells became full of dead cells, caused by viral propagation, and the debris was screened for proper protein expression by immunostaining and immunoblotting with the anti-FLAG antibody M2. We obtained Adv-CA-Fli, Adv-CA-FL1, Adv-CA-ΔN101f, Adv-CA-ΔN157f, Adv-CA-ΔN244f, Adv-CA-ΔN346f, which expressed different forms of p120ctn proteins under the control of the CAG promoter (Niwa et al., 1991), and Adv-CA-N120 for analysis. The full-length N-cadherin expression vector Adv-Ncad was described previously (Nakagawa and Takeichi, 1998). The recombinant adenoviruses were amplified, and purified by CsCl gradient centrifugation (Kanehara et al., 1994). FCS was added to the purified adenovirus solutions at a final concentration of 10%. Aliquots of the virus solution were stored at −80°C until used.

**Cell Extraction and Immunoprecipitation**

Colo 205 cells were removed from dishes by pipetting, rinsed twice in HBSS, and lysed in TBS-Ca (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM CaCl₂) supplemented with 1% Triton X-100, 1% NP-40, 1 mM PMSF, 1 mM NaVO₃, and 50 mM NaF (TBS-Triton) for 30 min at 4°C on a rocking platform. The soluble fraction was collected by centrifugation and precipitated with 50 μl of secondary antibody-conjugated beads and 5% BSA on ice for 20 min. After precipitation, incubations with primary and secondary antibodies were sequentially performed on ice for 1 h each, followed by washing three times with TBS-Triton. Finally, samples were boiled in 150 μl of SDS sample buffer with 7.5 μl of β-mercaptoethanol for 5 min.

**Gel Electrophoresis, Immunoblotting, and Immunohistochemistry**

Proteins were separated in SDSPAGE and electrophoretically transferred to membranes. The transferred proteins were detected by the enhanced chemiluminescence system (RPN 2106; Nycomed-Amschram). Immunohistochemical detection of cadherin and catenins was performed as described in Watabe-Uchida et al. (1998).

**Phosphatase Treatment**

Immunoprecipitated materials were washed twice with phosphatase reaction buffer (50 mM Tris-HCl, pH 8.5, 50 mM MgCl₂, 1 mM PMSE, 1 mM DTT, 0.1% Triton X-100, 0.1% NP-40, 1 mM PMSE, 1 mM NaVO₃, and 50 mM NaF (TBS-Triton)) for 30 min at 4°C on a rocking platform. The soluble fraction was collected by centrifugation and precipitated with 50 μl of secondary antibody-conjugated beads and 5% BSA on ice for 20 min. After precipitation, incubations with primary and secondary antibodies were sequentially performed on ice for 1 h each, followed by washing three times with TBS-Triton. Finally, samples were boiled in 150 μl of SDS sample buffer with 7.5 μl of β-mercaptoethanol for 5 min.

**Phosphorothioate Metabolic Labeling and Phosphoamino Acid Analysis**

For 32P-metabolic labeling, cells were cultured in phosphate-free DME (#21097-035; Life Technologies Inc.) with 0.3 μCi of 32P (NEX-053, New England Nuclear Life Science Products, Inc.) for 24 h. Then, cells were harvested and trypsinized when necessary. From their detergent extracts prepared as above, p120ctn was immunoprecipitated and separated by SDSPAGE. From the gels, the labeled p120ctn-protein band was excised after comparing with their autoradiograms, and the collected gel pieces were homogenized in 500 μl of freshly prepared 50 mM NH₄HCO₃. After addition of 25 μl β-mercaptoethanol and 5 μl 10% SDS, the samples were boiled for 5 min and agitated at room temperature for 2 h. Supernatants were collected after centrifugation at 15,000 rpm for 5 min. With 20 μg RNase A and 250 μl ice-cold TCA (100% wt/wt), and incubated on ice for 1 h. After centrifugation, the pellets were air-dried and proteins were digested by incubating with 50 μl 6 N HCl at 110°C for 90 min. Digested products were air-dried again and resuspended in buffer (2.2% formic acid, 7.8% glacial acetic acid), pH 1.9, with unlabeled phosphoserine, phosphothreonine, and phosphotyrosine. Samples were spotted on TLC plate and separated by two-dimensional electrophoresis at 1.5 kV for 40 min with the pH 1.9 buffer, and at 1.0 kV for 30 min with buffer (5% glacial acetic acid, 0.5% pyridine), pH 3.5. Finally, labeled phosphoamino acids were visualized by the BAS-1000 image analyzing system (FUJIX Inc.).

**Results**

**Induction of Compact Aggregate Formation in Colo 205 Cells**

Colo 205 cells grow as dispersed cells occasionally forming loose, small clusters (Fig. 1A) as seen in catenin-deficient cells (Hirano et al., 1992; Oyama et al., 1994; Shimoyama et al., 1992), and they only lightly attach to the culture dish. Despite this behavior, they express an apparently normal set of E-cadherin, αE-catenin, β-catenin, and p120ctn proteins (Fig. 1E). Moreover, the expression levels of these proteins are similar to those in HT-29 cells, which can organize epithelial sheets (see Fig. 7A). To determine whether the cadherin–catenin system in Colo 205 cells is entirely inactive, we rotated the cultures to facilitate cell aggregation. Under these culture conditions, Colo 205 cells clumped into larger aggregates (Fig. 1C) and this clumping was inhibited by addition of the E-cadherin.
blocking antibody SHE78-7 (Fig. 1 D), indicating that E-cadherin is functional. However, their aggregates were still loose and never formed tightly associated compact aggregates as generally produced by the cadherin adhesion system (Takeichi, 1988). These observations suggest that Colo 205 cells expose functional E-cadherin molecules on their surface, but their cadherin system has some deficit in exerting its full activity to organize compact cell aggregates. Interestingly, when the cultures of Colo 205 cells were starved by not refreshing the culture medium for a few days, they occasionally formed compact aggregates (Fig. 1 B), implying that their cadherin system is reversibly impaired and can be reactivated under certain physiological conditions.

As an initial attempt to investigate how the cadherin system is impaired in Colo 205 cells, we examined the effect of various biochemical reagents, including kinase inhibitors and activators, on their aggregation. Among the reagents tested, staurosporine showed a marked effect. It induced compact cell clustering in Colo 205 cultures within a few hours after administration (Fig. 2, A–D), during which the initial sign of adhesion induction was observed within 2 h. This effect was saturated by 6 h. The compaction of cell clusters was E-cadherin–dependent, as it was inhibited by SHE78-7 (Fig. 2 E). Staurosporine also slightly promoted spreading of cells, but this was not inhibited by SHE78-7 (Fig. 2 E, arrows). Since staurosporine is known to inhibit protein kinase C (PKC), we tested other PKC inhibitors, but none were effective (Table I). Tyrosine kinase inhibitors were also negative, except herbimycin A showed a weak compaction-inducing activity, but only when cells were cultured overnight with this inhibitor (data not shown). PKC activators and phosphatase inhibitors, such as phorbol esters and okadaic acid, also had no effects (data not shown). Thus, among the reagents tested, staurosporine exhibited an exceptionally strong effect on Colo 205 aggregation. This effect of staurosporine was reversible as its removal caused redispersion of Colo 205 cells (data not shown).

Besides metabolic inhibitors, we found that trypsin is a strong inducer for compact Colo 205 aggregate. When low concentrations of trypsin (0.01–0.001%) were added to serum-free cultures, Colo 205 cells became tightly associated with each other, deforming their morphology (Fig. 2, F–H). This adhesion induction was quick, beginning within 15 min after the addition of trypsin, and the effect was almost saturated at 30 min. The trypsin-mediated induction of aggregation was completely blocked by SHE78-7 (Fig. 2 I), indicating that it was an E-cadherin–dependent process. Under these conditions, which included a physiological concentration of Ca$^{2+}$, cadherins are not digested with trypsin (Takeichi, 1977). Trypsin concentration of 0.001% was sufficient for the above adhesion induction, whereas 0.0001% was not effective. Thus, we found two different classes of reagents to induce compact Colo 205 aggregation, trypsin as a quicker inducer and staurosporine as a slower inducer.

**Localization of MUC1, E-Cadherin, and Catenins in Colo 205 Cells**

Previous studies indicated that mucins, such as MUC1 (episialin; Wesseling et al., 1996; Kondo et al., 1998) and epiglycanin (Kempelen et al., 1994), reduce cell–cell adhesion presumably by steric hindrance. As Colo 205 cells express MUC1 (Baeckstrom et al., 1991), trypsin may remove such antiadhesive mucins. To check this possibility, we immunofluorescently stained for MUC1 in cells treated with (K) or without (J) 0.001% trypsin. MUC1 can be detected in both samples, even at cell–cell boundaries (arrows in K). Bars, 40 μm (A–I); 20 μm (J and K).
ter trypsin treatment, and found that this proteoglycan was equally present on the surface of both trypsinized and untrypsinized cells (Fig. 2, J and K), even being localized at intercellular contact sites in the adhesion-induced aggregates. Treatment of cells with O-sialoglycoprotein endopeptidase, which inactivates epiglycanin and enhances adhesion of mammary carcinoma cells (Kemperman et al., 1994), showed no effects on Colo 205 aggregation (data not shown). From these observations, we assumed that the effect of trypsin was not to remove steric hindrance molecules, but to digest some signaling proteins on the cell surface, such as receptors, affecting intracellular physiological states. This idea is supported by results of other experiments described below.

Immunostaining for E-cadherin in Colo 205 cells show a diffuse distribution of this molecule on their surface (Fig. 3, A and A’). When compaction was induced in their aggregates by staurosporine or trypsin treatment, E-cadherin became highly concentrated into cell–cell contact sites (Fig. 3, C, C’, E, and E’). Catenins displayed a similar distribution (see p120ctn in Fig. 3, B, B’, D, D’, F, and F’). These observations suggest that the E-cadherin–catenin complex can be redistributed to cell–cell contact sites under the above compaction-inducing conditions.

Changes in Electrophoretic Mobility of p120ctn

To study if any changes were induced in the cadherin–catenin system following the above treatments, we immunoprecipitated E-cadherin from Colo 205 cells untreated or treated with staurosporine or trypsin. The amount of catenins coprecipitating with E-cadherin was not changed after these treatments (Fig. 4 A). However, we noted a significant change in the pattern of the p120ctn band. In untreated Colo 205 cells, p120ctn was detected as a broad, diffuse band that appeared to comprise multiple components (Fig. 4 A, lanes p120 c), although the pattern varied slightly from experiment to experiment. After compaction-inducing treatments, the band pattern was altered. Its most intense portion was shifted to the position corresponding to the bottom (electrophoretic front) of the original band (Fig. 4 A, lanes p120, t and s). Similar electrophoretic patterns of these proteins were observed in Western blots of the whole cell lysates (Fig. 4 B). The changes in the p120ctn band pattern coincided with the onset of adhesion induction by each reagent, starting within

Table I. Effect of Protein Kinase Inhibitors on Aggregation of Colo 205 Cells

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
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<tr>
<td>Staurosporine</td>
<td>35 nM 15 nM 7.0 nM 3.5 nM 0.7 nM</td>
</tr>
<tr>
<td>UCN-01</td>
<td>100 nM 50 nM 10 nM 5 nM 1 nM</td>
</tr>
<tr>
<td>Calphostin C</td>
<td>200 nM 100 nM 50 nM 10 nM 5 nM</td>
</tr>
<tr>
<td>Herbimycin A</td>
<td>2 µg/ml 1 µg/ml 0.5 µg/ml 0.2 µg/ml 0.1 µg/ml</td>
</tr>
<tr>
<td>Tyrphostin</td>
<td>150 µM 75 µM 30 µM 15 µM 7.5 µM</td>
</tr>
<tr>
<td>Genestein</td>
<td>78 µM 39 µM 26 µM 13 µM 6.5 µM</td>
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</table>

Cells were cultured in the presence of each inhibitor for 6 h, and their morphological changes were then observed. +, cell adhesion was induced; −, not induced. Herbimycin A was not effective at the end of the 6-h incubation, but it induced adhesion after 24 h. All others, except staurosporine, showed no effect even after the 24-h incubation. The specificity and concentrations of each inhibitor blocking 50% of enzymatic activity are as follows: staurosporine, 2.7 nM for PKC, 8.2 nM for PKA, and 6.4 nM for p60<sup>v-src</sup>; UCN-01, 4.1 nM for PKC, 42 nM for PKA, and 45 nM for p60<sup>v-src</sup>; calphostin C, 50 nM for PKC, >50 µM for PKA, and p60<sup>v-src</sup>; herbimycin A, 5 µg/ml for p60<sup>v-src</sup> and p60<sup>v-abl</sup>; tyrphostin, 15 µM for EGF receptor; genestein, 22.6 µM for EGF receptor, and 25.9 µM for p60<sup>v-src</sup>.

Figure 3. Localization of E-cadherin and p120<sup>ctn</sup> in Colo 205 cells. (A and B) Control Colo 205 cells immunostained for E-cadherin (A) and p120<sup>ctn</sup> (B). (C-F) Cells treated with 7 nM staurosporine for 6 h (C and D) or with 0.001% trypsin for 30 min (E and F), and immunostained for E-cadherin (C and E) or p120<sup>ctn</sup> (D and F). (A’-F’) Phase-contrast images of A-F. Bar, 20 µm.
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Figure 4. Analyses of E-cadherin and catenins before and after adhesion induction. (A) Immunoblot analysis of E-cadherin-catenin complexes. E-Cadherin was immunoprecipitated from Colo 205 cells treated with 0.001% trypsin for 30 min (t) or from untreated control cells (c), and copurified catenins were detected in each panel as indicated. Note the band shift in p120ctn after trypsin treatment but no changes in the other catenins. A similar result was obtained from cells treated with 7 nM staurosporine (s) for 6 h. (B) Immunoblot detection of E-cadherin and catenins from a whole lysate of treated cells as in A. E-Cad, E-cadherin; α-cat, α-catenin; β-cat, β-catenin; p120, p120ctn. (C and D) Time-dependent changes in the p120ctn band pattern after trypsin (C) or staurosporine (D) treatment. Lane 30 min + mAb, cells were incubated with trypsin in the presence of SH E78-7, an antibody to block E-cadherin, for 30 min; lane trypsin removed, trypsin was removed after 30 min, and then cells were further cultured overnight. Whole cell extracts were loaded. (E) p120ctn-band shift after treatment with 0.01% trypsin in the presence (TC) or absence (TE) of 1 mM Ca2+. The band pattern was similarly changed after both treatments. E-Cadherin was left intact in TC-treated cells, but digested in TE-treated cells. α cat, α-catenin; β cat, β-catenin; p120, p120ctn. (F) Time-dependent changes in the p120ctn band pattern after PPase treatment of cells. Lane 30 min + mAb, cells were incubated with trypsin in the presence of SH E78-7, an antibody to block E-cadherin, for 30 min; lane trypsin removed, trypsin was removed after 30 min, and then cells were further cultured overnight. Whole cell extracts were loaded. (F) p120ctn-band shift after treatment with 0.01% trypsin in the presence (TC) or absence (TE) of 1 mM Ca2+. The band pattern was similarly changed after both treatments. E-Cadherin was left intact in TC-treated cells, but digested in TE-treated cells. α cat, α-catenin; β cat, β-catenin; p120, p120ctn. (G) Time-dependent changes in the p120ctn band pattern after PPase treatment of cells. Lane 30 min + mAb, cells were incubated with trypsin in the presence of SH E78-7, an antibody to block E-cadherin, for 30 min; lane trypsin removed, trypsin was removed after 30 min, and then cells were further cultured overnight. Whole cell extracts were loaded.

We sought to understand the molecular nature of the p120ctn-band shift. When p120ctn immunoprecipitates collected from untreated Colo 205 were incubated with alkaline phosphatase, the diffuse p120ctn band was transformed into a sharp band positioned at the front of the original (Fig. 4 F), which comigrated exactly with the p120ctn from staurosporine or trypsin treated cells. This suggests that the band shift observed may have been brought about by p120ctn dephosphorylation also. We then analyzed phosphorylated residues in p120ctn by 32P-metabolic labeling. The results showed that the major phosphorylated residues were serine (Fig. 4 G). However, their labeling intensity was however, not significantly reduced after trypsin treatment of cells. Anti-phosphotyrosine antibodies detected only weak signals from the p120ctn bands in these cells (data not shown). It is possible that the p120ctn-band shift is caused by dephosphorylation of a subset of the phosphorylated residues. Alternatively, the quantitative differences in phosphorylation suggested by the phosphatase treatments may be underrepresented by amino acid analysis methodology.

**Truncated p120 Induces Compaction in Cell Aggregates**

The above findings suggest that the process of p120ctn-band shift could be associated with the induction of compact Colo 205 aggregation. To pursue this possibility, we attempted to modify the activity of p120ctn by expressing a series of its deletion constructs attached to a FLAG tag at the COOH terminus in Colo 205 cells (Fig. 5 A).

p120ctn was immunopurified, and separated by SDS-PAGE. (Right) PAA analysis. The p120ctn bands in the left panel were excised, and PAA s were separated by two-dimensional electrophoresis. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. Molecular weight markers in A and B, 116 and 97.4 x 10^3.
expression of these molecules was achieved by electroporation of cDNAs or infection by adenoviral expression vectors. Generally, the adenoviral infection yielded much higher cDNA transfection efficiencies. Successfully transfected cells were detected by immunofluorescence staining with antibodies to the FLAG-tag. We first tested the full-length p120<sup>ctn</sup> as a control and found no particular effect on the aggregation of Colo 205 cells (Fig. 6 A). Then, we transfected them with ∆N346f, leaving the Armadillo repeat domain intact. Notably, cell groups expressing this construct were transformed into tightly associated aggregates in which the introduced molecules were sharply concentrated at cell–cell contact sites (Fig. 6 B). Then we tested other deletion constructs. Concerning shorter NH<sub>2</sub>-terminal deletions, ∆N323f displayed the same adhesion-inducing effect as ∆N346f (Fig. 6 C), and ∆N244f also showed a positive effect, but their aggregation appeared looser than that observed with the former (Fig. 6 D). On the other hand, ∆N157f and ∆N101f had no effect on cell adhesion (Fig. 6, E and F). These observations indicate that the critical sites are located between 245 and 323. We tested two other constructs, FLΔArf and ∆N346ΔArf, in which the Armadillo repeat domain was partially deleted, and found that they had no effect on cell aggregation (Fig. 6, G and H). The last two molecules did not bind to E-cadherin, as previously found (Daniel and Reynolds, 1995; data not shown), whereas all the others tested did. Consistently, FLΔArf and ∆N346ΔArf were distributed only in the cytoplasm (Fig. 6, G’ and H’) whereas the others were located along the cell membrane, as well as the cytoplasm (Fig. 6, A’–F’). Cell membrane distributions were categorized into two groups: the adhesion-inducing mutant molecules were concentrated into cell–cell contact sites (e.g., Fig. 6, B’ and C’, arrows) whereas the noneffective proteins were located randomly along the cell membrane.

In the preceding experiments, the p120<sup>ctn</sup>-band shift was correlated with adhesion induction. Therefore, we also analyzed the band profile of the NH<sub>2</sub>-terminus deletion constructs. In Western blotting of the lysates of cells transfected with these constructs, all NH<sub>2</sub>-terminally deleted molecules showed broad electrophoretic bands (Fig. 5 B), although the proportion of the bottom to the upper components in the band tended to increase in the adhesion-inducing construct ∆N346f. When FLf and ∆N346f were collected by immunoprecipitation and treated with phosphatase, only the bottom component remained undigested in both samples (Fig. 5 C), as found in endogenous p120<sup>ctn</sup>, indicating that the bottom bands of different p120<sup>ctn</sup> constructs were equivalent to each other in terms of phosphatase resistance.

We examined whether or not the binding of p120<sup>ctn</sup> to E-cadherin was altered by NH<sub>2</sub>-terminal deletions. We transfected Colo 205 cells with FLf or mutant ∆N346f, and immunoprecipitated E-cadherin from them (Fig. 5 D). Anti-FLAG antibody D8 detected only the ectopic molecules. The antibodies recognizing the COOH-terminal region of p120<sup>ctn</sup> detected both the transfected and endogenous molecules (Fig. 5 D, middle and right panels). Comparison of the band profiles in these samples with those in Western blotting of whole cell lysates (Fig. 5 E) indicates that the proportion of the E-cadherin–bound p120<sup>ctn</sup> to its entire pool was not different between FLf and ∆N346f. The small difference seen in electrophoretic mobility between the endogenous and full-length ectopic molecules is probably due to their difference in species origin, the former from the mouse and the latter from the human.
These results suggest that the binding affinity of p120ctn for E-cadherin was not altered by the NH2-terminal deletion.

**p120ctn in Other Cell Lines**

We tested two other epithelial lines, HT-29 and MDCK cells, to ask whether they also respond to the NH2-terminal–deleted p120ctn constructs, as well as the compaction-inducing reagents. Human colon carcinoma HT-29 cells, which express normal levels of E-cadherin and catenins (Fig. 7 A), organize into epithelial sheets in confluent cultures (Fig. 7 B, c; Fogh and Trempe, 1975; Chantret et al., 1988). When these cells are dispersed with trypsin in the absence of Ca2+ and seeded into new plates they require a lag period to reestablish epithelial sheets, e.g., at 18–24 h after cell transfer, the cultures still contain many round, dispersed cells (Fig. 7 B, b), although they eventually established confluent epithelial sheets at 48 h. We analyzed p120ctn in these HT-29 cells, and found that its band pattern dynamically changed with the cycle of cell transfer. p120ctn derived from freshly trypsinized HT-29 cells showed a single band (Fig. 7 F). 24 h after the transfer p120ctn was shifted to an upper position (Fig. 7 F). At 48 h, when cells formed confluent epithelial sheets, the p120ctn band returned to the lowest position (Fig. 7 F). We tested if the p120ctn-band shift observed after overnight culture could be canceled by retreatment with trypsin (0.001%; 30 min) or staurosporine (7 nM; 6 h) by adding them to HT-29 cells precultured for 18 h. The results showed that both treatments abolished the mobility shift of the p120ctn band (Fig. 7 G). The range of the p120ctn-band shifting was similar to that found in Colo 205 cells. In correlation with this p120ctn band change, tight cell–cell association was induced in HT-29 cells precultured for 18 h and then incubated with staurosporine (7 nM) for 6 h or with trypsin (0.001%) for 30 min (Fig. 7 B, e and f), as seen in the case of Colo 205 cells.

On the other hand, MDCK, which is widely used as a model of polarized epithelial cells, reorganized epithelial sheets within 18 h after transfer. Addition of staurosporine or trypsin to such MDCK cultures had no effects on cell–cell association (Fig. 7 C), although we observed subtle morphological alterations in the treated cultures. A analysis of p120ctn in MDCK cells detected two major bands as reported by Mo and Reynolds (1996), the upper band likely corresponds to the p120ctn band in human colon carcinoma lines (Fig. 7 F). The p120ctn pattern in MDCK was not changed by trypsin-mediated cell transfer. When MDCK cells were treated with staurosporine, the electrophoretic mobility of p120ctn was slightly enhanced (Fig. 7 G), as described by Ratcliffe et al. (1997). However, this position...
shift of the p120\textsuperscript{ctn} band was subtle compared with that observed in the above carcinoma lines. Trypsin treatment of MDCK cells did not affect the mobility of p120\textsuperscript{ctn} (Fig. 7 G).

Next, we transfected the above cell lines with the NH\textsubscript{2}-terminus-truncated ΔN346f, as well as with the control FLf immediately after cell transfer, and examined the effects after 24 h. ΔN346f, but not FLf, enhanced HT-29 cell–cell association (Fig. 7 D). ΔN346f-expressing cells formed compact aggregates, whereas FLf had no effects. Rather, we observed that high-level overexpressions of FLf tended to disperse epithelial sheets (data not shown). In MDCK cells, neither construct affected the cell–cell contact morphology, they simply accumulated at cell–cell contact sites (Fig. 7 E).

**Induction of Colo 205 Aggregation by N-Cadherin Lacking the p120\textsuperscript{ctn}-binding Site**

These findings suggest that in particular cells such as Colo 205, p120\textsuperscript{ctn} inhibits cadherin-mediated adhesion under certain physiological conditions. If this is the case, cadherin activity might be restored under circumstances where it cannot interact with p120\textsuperscript{ctn}. To test this possibility, we transfected Colo 205 cells with two different constructs of N-cadherin cDNA, one encoding its entire portion and the other encoding a mutant molecule in which the juxtamembrane region of the cytoplasmic domain, which is known to contain the p120\textsuperscript{ctn}-binding site (Yap et al., 1998), has been deleted (Fig. 8 A). Immunoprecipitation with anti-N-cadherin antibodies from the transfected cells confirmed that the mutant N-cadherin was unable to associate with p120\textsuperscript{ctn} (Fig. 8 B), but coprecipitated normally with β-catenin. Morphological and immunostaining examinations of these transfectants showed that the expression of full-length N-cadherin had no effect on cell–cell adhesion, suggesting that cadherins were generally blocked in the Colo 205 environment, irrespective of their type. In contrast, the mutant N-cadherin induced a strong aggregation of Colo 205 cells (Fig. 8 C). These findings suggest that cadherins can function normally in these cells unless they interact with p120\textsuperscript{ctn}, supporting the idea that p120\textsuperscript{ctn} can act as an inhibitor of cadherin function.

**Discussion**

This work was initiated to understand why Colo 205 cells cannot undergo typical E-cadherin–dependent association despite their normal expression of E-cadherin and associated proteins. We found that the treatment of these cells with two independent reagents, trypsin and staurosporine, could reactivate the E-cadherin adhesion system.
A t the initial mechanisms for their actions remain unknown, we assume that trypsin removes or truncates some cell surface proteins involved in intracellular signaling, leading to an activation or suppression of their downstream cascade and via this pathway, modulates the cadherin system. The response of Colo 205 cells to the trypsin treatment was rapid, suggesting that the proposed signaling system probably does not require transcription of new genes. A s another possible mechanism of the trypsin action, we considered that this enzyme might have facilitated cell adhesion by digesting such surface components as mucins, known to physically prevent cell–cell contacts. H owever, this possibility is less likely, because we could induce compact cell aggregation by intracellular manipulation of Colo 205 cells without enzymatic treatment. M oreover, no correlation was found between the distribution of M UC 1, a major Colo 205 mucin, and adhesion induction. C oncerning staurosporine, its action could be connected with the proposed signaling cascade directly or indirectly. O f the many kinase inhibitors tested, only staurosporine e ffectively induced Colo 205 cell adhesion within a short incubation period. T his suggests that staurosporine has a specific target in its action on Colo 205 adhesion, although we cannot specify it because this antibiotic can inhibit multiple classes of enzymes. It was reported that retinoic acid treatment results in a similar adhesion induction (Nakagawa et al., 1998), suggesting that this reagent is another effector to activate the cadherin system.

S ince the initial or intermediate steps of the proposed signaling system could be complex, we focused our analysis on its putative terminal step, asking if the cadherin–catenin complex per se was modified during the adhesion induction, and we identified an alteration in the electrophoretic mobility of p120ctn. T his change could be triggered in the absence of E-cadherin–mediated adhesion, implying that it resulted directly from the trypsin or staurosporine-triggered signaling cascade. A trypsin/staurosporine-sensitive mobility shift of p120ctn was also observed with another carcinoma line HT-29. A ough transient in these cells, the shift nonetheless correlated perfectly with adhesion induction. In contrast, MDCK cells neither showed such modulation of p120ctn, nor responded to the adhesion-inducing reagents. A ough staurosporine treatment slightly enhanced the electrophoretic mobility of p120ctn, even in MDCK cells (Ratcliffe et al., 1997), the magnitude of the band shifting in this case was much smaller as compared with that for Colo 205 and HT-29 cells.

T hese findings prompted us to further examine the role for p120ctn by structure-function analysis, and we found that NH2-terminally truncated p120ctn constructs by themselves could reactivate the E-cadherin system. T his observation implies that p120ctn inhibits cadherin-mediated adhesion in Colo 205 cells. T hus, we postulate that the NH2-terminally deleted p120ctn lacks this activity, and competes with endogenous molecules, ultimately resulting in the reactivation of the cadherin system. T his hypothesis is strongly supported by the finding that ectopic N-cadherin induced aggregation of Colo 205 cells only when it was unable to bind to p120ctn. T his finding also suggests that different types of cadherin, in general, cannot function in the intracellular environment of Colo 205 if they bind to p120ctn.

T he correlation between the band shifting of p120ctn and adhesion induction suggests that the putative inhibitory activity of this molecule might be elicited by certain biochemical modifications. In Colo 205 cells, the original p120ctn band was broad. A ough multiple splicing products of p120ctn are expressed in many carcinoma lines (Mo and Reynolds, 1996), the broad band observed in Colo 205 cells appears to result from phosphorylation at various levels because phosphatase treatment of the Colo 205 p120ctn resulted in the generation of a sharp single band. T his phosphatase-treated p120ctn was similar in electrophoretic mobility to that found in the adhesion-induced Colo 205 cells. T hese findings suggest the possibility that p120ctn acquires the inhibitory activity when hyperphosphorylated, and the staurosporine/trypsin-induced signals reduce the
level of phosphorylation. We found that the major phosphorylated residue in p120<sup>ctn</sup> of Colo 205 cells was serine, consistent with a previous observation by Ratcliffe et al. (1997). Our results do not exclude the possibility that other residues are also phosphorylated at low levels, as it has been reported that tyrosine is phosphorylated in p120<sup>ctn</sup> of other cell lines (Kinch et al., 1995; Calautti et al., 1998; Hazon and Norton, 1998; Rosato et al., 1998). The net amount of serine phosphorylation in p120<sup>ctn</sup> did not appear reduced after its electrophoretic mobility shift, although phosphoamino acid analysis may not adequately detect small changes in phosphorylation status. If dephosphorylation is indeed involved in this process, one possibility is that only a specific subset of phosphorylation sites are required for the p120<sup>ctn</sup> change in mobility. The NH<sub>2</sub>-terminally deleted p120<sup>ctn</sup>, which induced cell adhesion, still contained slower-migrating, phosphatase-sensitive components, although their proportion tended to be reduced. Possibly, the key phosphorylation sites directly affecting the electrophoretic mobility of p120<sup>ctn</sup> are located outside the NH<sub>2</sub>-terminal region. These observations also suggest that hyperphosphorylation may be insufficient to confer the adhesion inhibitory activity on p120<sup>ctn</sup> if its NH<sub>2</sub> terminus is absent. A model for the signaling cascade to regulate p120<sup>ctn</sup> phosphorylation has been proposed by Ratcliffe et al. (1997).

How does p120<sup>ctn</sup> inhibit cadherin function? It is interesting to note that the juxtamembrane domain of the cadherin cytoplasmic region where p120<sup>ctn</sup> binds can inhibit cell adhesion when overexpressed (Kinner, 1992), and also is required for clustering of cadherin molecules (Yap et al., 1998) which is thought to be essential for cadherin-mediated cell adhesion. A further report shows that deletion of a similar region in E-cadherin resulted in activation of this adhesion molecule, enhancing its lateral interactions in the cell membrane of a leukemia line (Ozawa and Kemler, 1998b), suggesting that this domain can inhibit lateral clustering of cadherins. p120<sup>ctn</sup> is the only molecule known to bind to the juxtamembrane domain of cadherins and could be the major effector of these activities. For example, p120<sup>ctn</sup> may directly regulate lateral clustering of cadherins and this activity may be blocked by hyperphosphorylation of p120<sup>ctn</sup> or other components, although their proportion tended to be reduced. Possibly, the key phosphorylation sites directly affecting the electrophoretic mobility of p120<sup>ctn</sup> are located outside the NH<sub>2</sub>-terminal region. These observations also suggest that hyperphosphorylation may be insufficient to confer the adhesion inhibitory activity on p120<sup>ctn</sup> if its NH<sub>2</sub> terminus is absent. A model for the signaling cascade to regulate p120<sup>ctn</sup> phosphorylation has been proposed by Ratcliffe et al. (1997).

Identification of components of such cascades is an important future issue for unraveling the morphogenetic regulatory mechanisms of cell–cell adhesion.


