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Transmembrane control of cadherin-mediated cell adhesion: a 94 kDa protein functionally associated with a specific region of the cytoplasmic domain of E-cadherin

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Cadherins are a family of transmembrane glycoproteins which play a key role in Ca^{2+}-dependent cell-cell adhesion. Cytoplasmic domains of these molecules are anchored to the cell cytoskeleton and are required for cadherin function. To elucidate how the function of cadherins is controlled through their cytoplasmic domains, we deleted five different regions in the cytoplasmic domain of E-cadherin. After transfecting L cells with cDNA encoding the mutant polypeptides, we assayed aggregating activity of these transfectants; all these mutant proteins were shown to have an extracellular domain with normal Ca^{2+}-sensitivity and molecular weight. Two mutant polypeptides with deletions in the carboxy half of the cytoplasmic domain, however, did not promote cell-cell adhesion and had also lost the ability to bind to the cytoskeleton, whereas the mutant molecules with deletions of other regions retained the ability to promote cell adhesion and to anchor to the cytoskeleton. Thus, the cytoplasmic domain contains a subdomain which was involved in the cell adhesion and cytoskeleton-binding functions. When E-cadherin in F9 cells or in L cells transfected with wild-type or functional mutant cadherin polypeptides was solubilized with nonionic detergents and immunoprecipitated, two additional 94 and 102 kDa components were coprecipitated. The 94 kDa component, however, was not detected in the immunoprecipitates from cells expressing the mutant cadherins which had lost the adhesive function. These results suggest that the interaction of the carboxy half of the cytoplasmic domain with the 94 kDa component regulates the cell binding function of the extracellular domain of E-cadherin.

Introduction

Cadherins are a family of transmembrane glycoproteins which function for Ca^{2+}-dependent cell-cell adhesion (Takeichi, 1988). Amino acid sequences are conserved among different types of cadherins in a range of 43–58%; the degree of conservation, however, varies with the region of the molecules (Hatta *et al.*, 1988). The most highly conserved region is the carboxy (C-) terminal cytoplasmic domain, although the cell binding function, the principal function of this molecular family, resides in the amino (N-) terminal extracellular domain. It should thus be intriguing to understand the role of the cytoplasmic domain in cadherin function.

Cadherins present in plasma membranes are divided into two populations, one soluble and the other insoluble to nonionic detergents (Hirano *et al.*, 1987). Immunostaining studies showed that the insoluble cadherins coincide with the cortical actin bundles localized at cell-cell boundaries (Hirano *et al.*, 1987). These observations suggest that the insoluble population of cadherins are anchored to actin-based cytoskeletons. In some cell types, cadherins are concentrated in the adherence-type cell-cell junctions which are known to be associated with cortical actin belts (Boller *et al.*, 1985; Volk and Geiger, 1986a,b; Takeichi, 1988).

Recent studies demonstrated that E-cadherin, a subclass of cadherins that is also called uvomorulin (Ringwald *et al.*, 1987), lost its cell binding activity when the C-terminus was truncated to various extents, although the extracellular domain of these mutant molecules was preserved intact (Nagafuchi and Takeichi, 1988; Ozawa *et al.*, 1989). These mutant E-cadherin molecules also lost the ability to bind to cytoskeletons. These results, together with the above observations, suggest that cadherins form a complex with some actin-based cytoskeletons through their cytoplasmic domain, and molecular interaction within the complex is necessary for the cell-binding function of the extracellular domain.

It has been reported that some proteins are coprecipitated with E-cadherin in immunoprecipitation of the detergent-soluble fraction of this molecule (Vestweber and Kemler, 1984; Peyrieras

et al., 1985; Vestweber *et al.*, 1987; Ozawa *et al.*, 1989). In the present study, we examined whether these proteins are implicated in the function of the cytoplasmic domain of E-cadherin. We isolated L cells expressing intact or mutant E-cadherin molecules with deletions in different positions of the cytoplasmic domain and examined their cell-binding ability as well as their association with other proteins. Here, we report that the carboxy half of the cytoplasmic domain is essential for the cell-binding function of E-cadherin and that a 94 kDa protein binds to E-cadherin through this region of the cytoplasmic domain. A possible role of this protein in cadherin-mediated cell adhesion is discussed.

Results

Isolation of cell lines with mutant E-cadherin polypeptides

To assess the role of different regions of the cytoplasmic domain of E-cadherin, we constructed cDNAs which encode five different deletion mutants of E-cadherin, designated EM20, EM21, EM32, EM33, and EM34 (Figure 1). In EM20 and EM21, 7 and 37 amino acid residues were deleted from the C-terminus, respectively. In EM32, 36 amino acid residues were deleted between position 814 and 849; in EM33, 40 residues were deleted between position 774 and 813; and in EM34, 23 residues were deleted between position 751 and 773 (see Nagafuchi *et al.*, (1987) for the numbering of the amino acid residues in E-cadherin). These cDNAs were connected to the β-actin and HSV-tk tandem promoter (McKnight, 1980; Fregien and Davidson, 1986) to construct the expression vectors shown in Figure 1, and these vectors were introduced into L cells together with a plasmid for neomycin resistance.

After G418 selection, a number of stable transfectants expressing polypeptides recognized by antibodies to E-cadherin were isolated. Of these, five clones designated ELβ20, ELβ21, ELβ32, ELβ33, and ELβ34 were chosen for further experiments, expressing the polypeptides encoded by EM20, EM21, EM32, EM33, and EM34, respectively. As a control cell line expressing normal E-cadherin polypeptides, ELs4 was used; this was obtained by the transfection of L cells with an expression vector encoding the full length cDNA for E-cadherin and has been described (Nagafuchi and Takeichi, 1988). ELβ21 is the same one as had been isolated previously (Nagafuchi and Takeichi, 1988).

Characterization of mutant E-cadherin polypeptides expressed on the transfectants

Immunoblot analysis showed that the different transfectant lines expressed E-cadherin polypep-

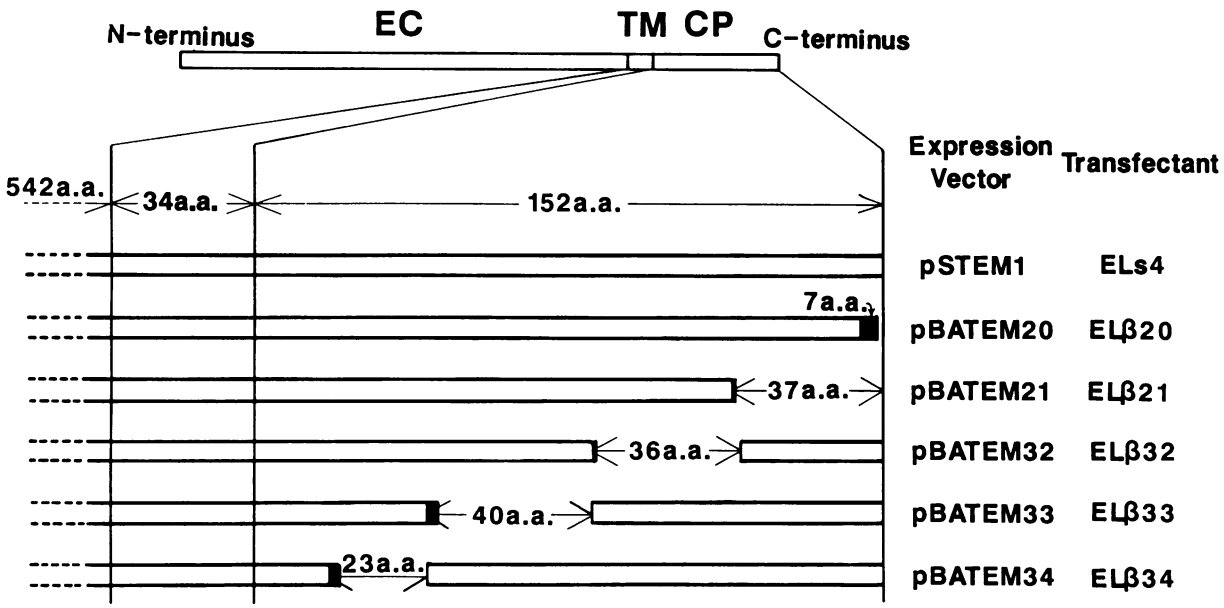

Figure 1. **Schematic diagram of mutant E-cadherin polypeptides with deletions in the cytoplasmic domain.** pSTEM1 is an expression vector for normal E-cadherin protein (Nagafuchi *et al.*, 1987). pBATEM20, pBATEM21, pBATEM32, pBATEM33, and pBATEM34 were the vectors for expression of the corresponding cDNAs. The right column shows the designation for the cell lines transfected with each plasmid. Solid boxes represent 1 to 4 amino acid residues added to the mutant proteins as a result of the constructions. EC, TM, and CP represent the extracellular domain, the transmembrane domain, and the cytoplasmic domain of the mature form of E-cadherin, respectively.

tides with different mobilities in SDS-gels, as expected (Figure 2A). The mobility of these mutant polypeptides, however, was not completely proportional to their putative molecular weight; e.g., the EM33 polypeptide showed a lower mobility than expected. Probably, different regions of E-cadherin have different effects on the electrophoretic mobility of this protein. In this regard, it should be noted that the molecular weight of cadherins estimated by electrophoretic mobility does not accord with that determined by amino acid sequence (Gallin *et al.*, 1987).

Normal cadherins are Ca^{2+}-sensitive: they can be digested with trypsin in the absence of Ca^{2+} but not in the presence of Ca^{2+} when live cells are treated. To examine whether the mutant cadherins retained this property, they were subjected to trypsin treatment in the presence and absence of Ca^{2+}. The results showed that all mutant molecules were Ca^{2+}-sensitive; i.e., they were degraded by trypsin only in the absence of Ca^{2+}. An example of these results is shown in Figure 2B.

It is also known that the treatment of the membrane fraction of cells with trypsin in the presence of Ca^{2+} causes release of an 84 kDa fragment of E-cadherin that covers the almost entire length of the extracellular domain of this molecule (Hyafil *et al.*, 1980; Cunningham *et al.*, 1984; Shirayoshi *et al.*, 1986). 84 kDa fragments were released from the membrane fraction of all the transfectant lines after trypsin-Ca^{2+} treatment (cf. Figure 2B), indicating that all of the mutant E-cadherin

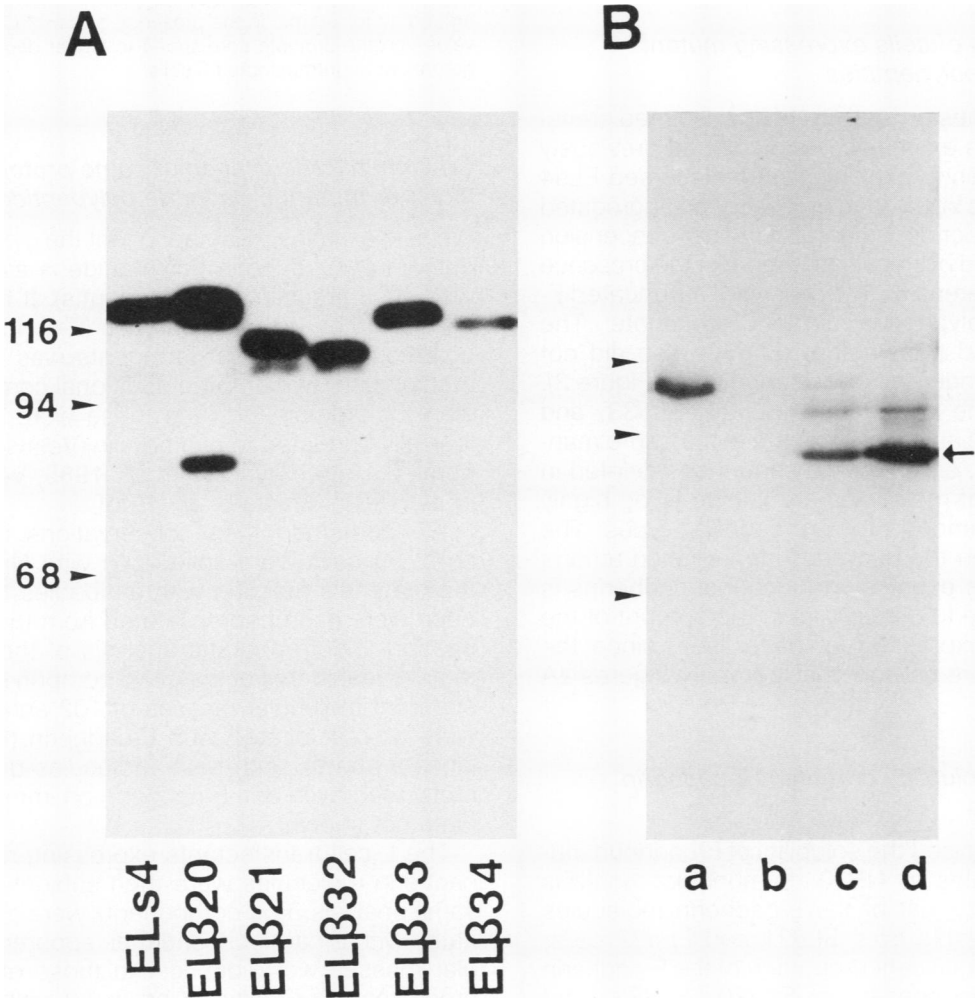

Figure 2. **Immunoblot detection of E-cadherin polypeptides in transfectants.** (A) E-cadherin polypeptides derived from different transfected cell lines. Samples obtained from 2×10^5 cells were loaded on each lane. Bands with smaller molecular mass seen in some lanes are probably degradation products. (B) Ca^{2+}-sensitivity of mutant E-cadherin polypeptides expressed in ELβ32 cells. Lanes a and b: E-cadherin polypeptide from cells treated with trypsin in the presence (a) or absence (b) of Ca^{2+}. Lanes c and d: 84 kDa E-cadherin fragments released by treatment of the membrane fraction of cells with trypsin in the presence of Ca^{2+}, obtained from ELβ32 cells (c) or control F9 cells (d). Samples extracted from 4×10^5 cells were loaded on each lane. Arrow indicates the position of the 84 kDa tryptic fragments. Arrowheads indicate the positions of molecular weight markers.

polypeptides expressed in these cell lines possess an extracellular domain with the same size as that of the normal molecule. It was also found that the extracellular domains of the mutant polypeptides retained the Ca^{2+}-sensitivity of the normal molecule, since the 84 kDa fragments derived from these molecules were degraded by treatment with trypsin in Ca^{2+}-free conditions (data not shown).

When live transfectant cells were immunostained with antibodies to E-cadherin, their surfaces reacted positively with the antibodies (data not shown). All of these results strongly suggest that the extracellular domains of the mutant molecules have the same properties as that of normal E-cadherin molecules and are exposed on cell surfaces.

Aggregation of cells expressing mutant E-cadherin polypeptides

Cell-cell adhesion activity of the isolated transfectants was examined. As described previously (Nagafuchi and Takeichi, 1988), dispersed ELs4 cells with the intact form of E-cadherin aggregated in a Ca^{2+}-dependent manner if the cell suspension was prepared by trypsin treatment in the presence of Ca^{2+}, whereas ELβ21 cells with a truncated E-cadherin polypeptide did not aggregate. The newly cloned transfectant, ELβ32, also did not aggregate under the above conditions (Figure 3). However, the other lines, ELβ20, ELβ33, and ELβ34, were able to aggregate in the same manner as ELs4, although the sequences deleted in these mutant molecules are known to be highly conserved among different cadherin types. The differences in the degree of aggregation among the cell lines expressing functional cadherins is probably due to differences in the amount of the molecules expressed in these lines, since the two values are proportional (compare Figures 2A and 3).

Detergent solubility of mutant E-cadherin polypeptides

We then analyzed the solubility of E-cadherin mutant molecules in NP40. Immunoblot analysis showed that most of the E-cadherin molecules were extractable from ELβ21 and ELβ32 cells, whereas a significant proportion of the E-cadherin molecules expressed in ELβ20, ELβ33, and ELβ34 cells was not (Figure 4). Thus, essentially all the nonfunctional molecules could be extracted with the detergent, whereas the functional molecules were only partially extractable. This result is consistent with the previous observations (Nagafuchi and Takeichi, 1988; Ozawa *et al.*, 1989).

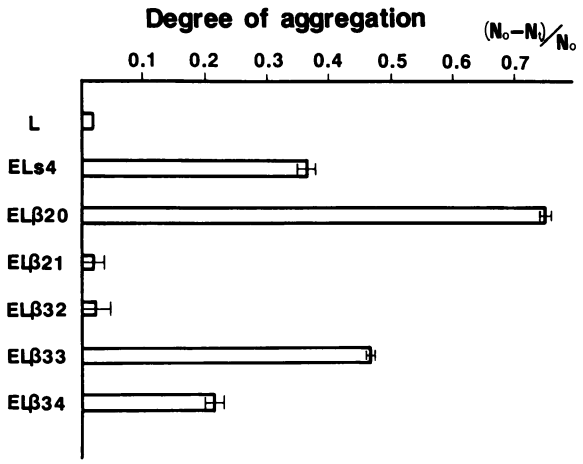

Figure 3. **Aggregation of cells with various E-cadherin polypeptides.** Cells dissociated by TC-treatment were allowed to aggregate for 30 min in the presence of 1 mM Ca^{2+}. Higher values on the ordinate correspond to higher degrees of aggregation. L, untransfected L cells.

Co-immunoprecipitation of some proteins with intact or mutant E-cadherin polypeptides

We next examined whether or not the cytoplasmic domain of E-cadherin polypeptide is associated with any intracellular component(s). It has been reported that, when uvomorulin (E-cadherin) solubilized into nonionic detergents was immunoprecipitated, two or three additional components were co-precipitated, and these were immunologically unrelated to uvomorulin (Vestweber and Kemler, 1984; Peyrieras *et al.*, 1985; Vestweber *et al.*, 1987; Ozawa *et al.*, 1989).

We confirmed these observations using F9 cells. F9 cells were solubilized with NP40, and the materials reacting with antibodies to E-cadherin were immunoprecipitated from the soluble fraction. Electrophoretic analysis of the precipitates revealed that at least two components, with apparent molecular masses of 102 and 94 kDa, were co-precipitated with E-cadherin (Figure 5, lanes a and b), and these molecules did not directly react with anti-E-cadherin on immunoblots (data not shown).

The L cell transfectants expressing normal E-cadherin molecules were then subjected to the same analysis. Two components were co-precipitated with E-cadherin, and their apparent molecular masses were identical to those of the co-precipitating proteins found in F9 cells. These molecules were also detected in immunoprecipitates from cells expressing the deletion mutants which retained the cell-binding activity. In contrast, in immunoprecipitates from cells expressing the deletion mutants which lost the cell-binding activity, the 94 kDa component was not detected

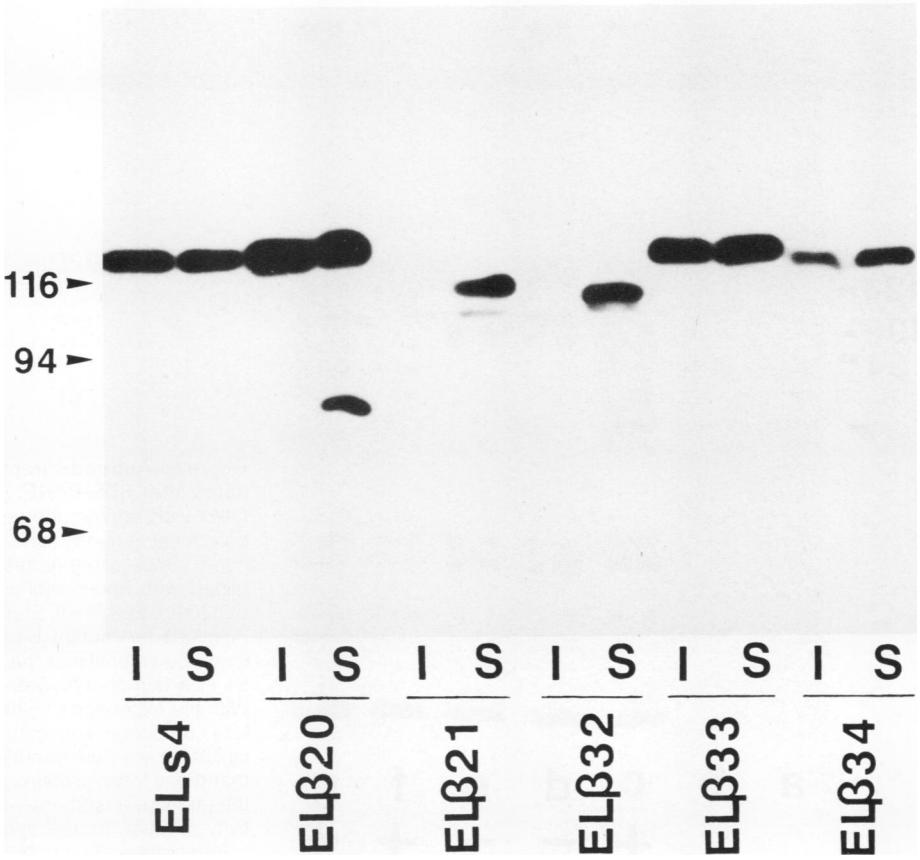

Figure 4. **Immunoblot analyses of detergent-insoluble (I) and soluble (S) fractions of E-cadherin polypeptides obtained from different transfectants.** Note that insoluble E-cadherin is nearly undetectable in ELβ21 and ELβ32. Samples derived from 4×10^5 cells were loaded on each lane, except that the sample for ELβ34 contained 8×10^5 cells per lane. Bands with smaller molecular mass in some lanes are probably products of degradation.

(Figure 5, lanes c–g). The 94 kDa component is thus associated only with functional E-cadherin polypeptides. On the other hand, the 102 kDa component appeared to be associated not only with the functional but also with the non-functional E-cadherin mutant polypeptides. Since the 102 kDa band on autoradiograms is relatively faint, this observation should be confirmed using more sensitive detection methods in future studies.

The 102 and 94 kDa proteins did not react with antibodies to α-actinin which has a similar molecular mass (data not shown).

Discussion

It has been reported that E-cadherin polypeptides whose C-terminus had been truncated lost the ability to mediate cell-cell binding (Nagafuchi *et al.,* 1988; Ozawa *et al.,* 1989). In the present study, we demonstrated that not only the deletion of the C-terminal 37 amino acid residues but also the deletion of 36 amino acids from the region adjacent to the above deletion position was effective in inactivating E-cadherin. On the other hand, deletions of other regions as well as the deletion of the C-terminal 7 amino acids did not cause the inactivation of the molecule. It is thus concluded that the region of 64 amino acids located close to the C-terminus, but not the terminus itself, contains the most important information for regulating the cell-binding function of the extracellular domain of E-cadherin. Seventy-five to 90% of amino acids are conserved among different cadherins in this region, supporting the notion of its functional importance.

It should be noted, however, that the other cytoplasmic regions whose deletion did not affect the E-cadherin activity are also highly conserved among different cadherins (Hatta *et al.,* 1988), implying that these regions also have some important functions. Possibly, these regions may not be essential for the cell-binding function itself of this molecular family, but they may participate in other regulatory processes of cadherin-mediated adhesion; e.g., such regions might be involved in the localization of cadherins at specific sites in the cell membrane, as it is known that cadherins tend to be concentrated on the apical side of cell-cell junctions in epithelial sheets. This kind of regulatory machinery may not exist in L cells and therefore can not be detected as long as model cells, such as L cell transfectants, are used for experiments. These results thus suggest a possibility that the cytoplasmic domain of cadherins

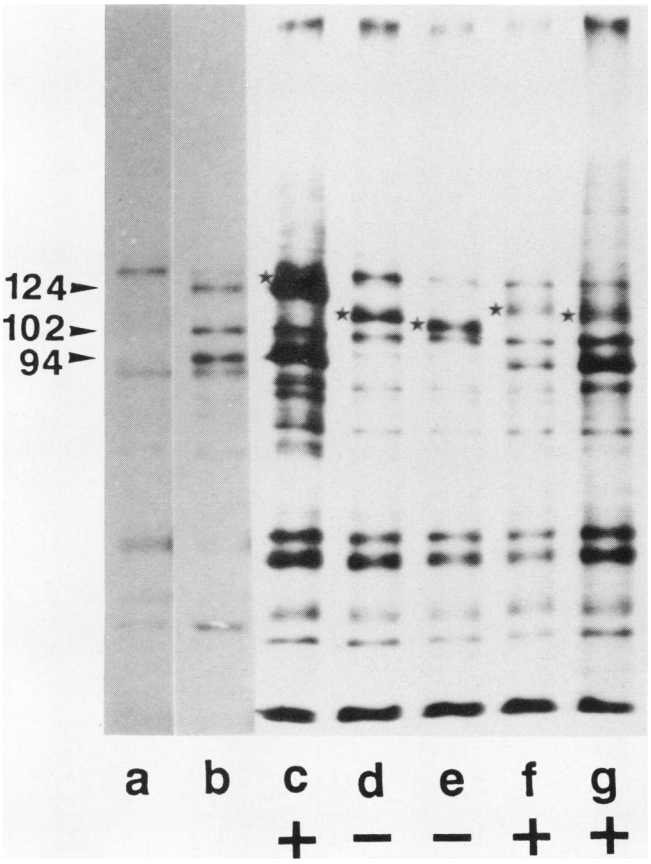

Figure 5. **Autoradiographic detection of immunoprecipitates after SDS-PAGE.** A lysate of F9 cells was precipitated with preimmune serum (lane a) or anti-E-cadherin polyclonal antibody (lane b). The 124 kDa band is E-cadherin. Lanes c to g represent the immunoprecipitates obtained with anti-E-cadherin from different transfectants: ELβ20 (c), ELβ21 (d), ELβ32 (e), ELβ33 (f), and ELβ34 (g). Positions for mutant E-cadherin polypeptides were indicated by asterisks at the left of each lane. Note that the 94 kDa component was not detected in lanes d and e. With the exception of E-cadherin and the 102 kDa and 94 kDa components, all bands were detected in immunoprecipitates using preimmune serum (not shown), indicating that these latter proteins are nonspecifically adsorbed on the immune adsorbents. Ca^{2+}-dependent cell binding activity of these transfectants is shown by + (adhesive) or − (nonadhesive) for comparison.

can be dissected into subdomains with different roles.

The present study confirmed previous observations that the cell-binding function of cadherins is associated with their ability to anchor to cytoskeletons and supports the idea that these two processes, which occur extra- and intracellularly, are functionally linked. Although the mechanism of such a link is unknown, we may postulate that the interaction of the cytoplasmic domain with the cytoskeleton may regulate the cell-binding function of the extracellular domain, or conversely, the extracellular cell-cell binding interaction between cadherins may send a signal to the cytoplasmic domain for regulating its binding to the cytoskeleton. In any case, an important role of the cytoplasmic domain should be to interact with intracellular components.

We have detected a 94 kDa protein which is associated only with functional E-cadherins. This protein seems not identical to already-characterized proteins, as judged by immuno-crossreaction experiments. How does this protein participate in the function of E-cadherin? A likely possibility is that the 94 kDa protein binds directly to the region adjacent to the C-terminus of E-cadherin, since deletion of this region resulted in no binding of this protein to E-cadherin. The bound 94 kDa protein may mediate the interaction between cadherins and actin-based cytoskeletons. With regard to the binding of the 94 kDa protein to E-cadherin, however, there is the alternative possibility that it may bind to some other region of E-cadherin, and this binding may be affected indirectly by the deletion of the C-terminal regions. Whichever the case is, the association of the 94 kDa protein is probably essential for the extracellular domain of E-cadherin to function as a cell-cell adhesion receptor.

The 94 kDa protein was detected not only in F9 cells but also in L cells, which have little endogenous cadherin activity. Although not proven, it is likely that the molecules detected in the two cell types are identical, because their apparent molecular mass is similar. If so, one might ask the question why a cadherin-binding protein exists in cells without cadherins. Perhaps, this protein functions not only for cadherins but also for some other systems. For further characterization of this molecule, its subcellular localization must be determined. A 102 kDa protein also binds to cadherins, but our data on this protein presented here are not sufficient for speculation about its role.

Similar observations on the association of pro-

teins with uvomorulin (E-cadherin) have recently been reported by Ozawa *et al.,* (1989). They found that three components were associated with uvomorulin, and the association of all of these components was lost when the C-terminus of this molecule was truncated. Probably, one of these components is identical to the 94 kDa protein detected in the present study.

In the adherence-type cell-cell junctions in which cadherins are a transmembrane component, it is known that a number of cytoplasmic components, such as vinculin, α-actinin, and actin form a complex (Geiger *et al.,* 1985). Recently, this complex was isolated from rat liver (Tsukita and Tsukita, 1989). At least 10 polypeptides, including vinculin, α-actinin, and actin, were detected in this preparation. Therefore, cadherin-mediated adhesion may depend on interactions among many kinds of cytoplasmic molecules. The 94 kDa protein is likely to be a component involved in such a molecular cascade.

Methods

Plasmid construction

Expression vectors pBATEM20, pBATEM21, pBATEM32, pBATEM33, and pBATEM34, which contained EM20, EM21, EM32, EM33, and EM34, respectively, were constructed from pBATEM2 (Nose *et al.,* 1988), which contains E-cadherin cDNA, covering the whole coding region, joined to the β-actin and HSV-tk tandem promoter (McKnight, 1980; Fregien and Davidson, 1986). Seven restriction sites (*Xho* I, *Bst*EII, *Sma* I, *Pvu* II, *Cla* I, *Sac* I, and *Rsa* I) in the open reading frame of the E-cadherin cDNA sequence and two other sites (*Eco*RI and *Xba* I) placed at the 3′ terminus of the cDNA were used to produce frame-shift mutants. The *Sma* I, *Pvu* II, *Cla* I, *Sac* I, and *Rsa* I sites are located in the coding region for the cytoplasmic domain; these sites correspond to the amino acid residues at positions 751, 773, 813, 848, and 878 (Nagafuchi *et al.,* 1987), respectively. pBATEM20 was constructed by replacing the *Bst*EII-*Cla* I fragment with the *Bst*EII-*Rsa* I fragment; in this case, the *Cla* I site was blunt-ended before replacement. To construct pBATEM32, the *Cla* I-*Eco*RI fragment was replaced by the *Sac* I-*Xba* I fragment; the *Sac* I and *Cla* I sites were blunt-ended and a *Xba* I linker was ligated to the blunt-ended *Eco*RI terminus. For the pBATEM33 and pBATEM34 constructions, the *Pvu* II-*Cla* I or the *Sma* I-*Pvu* II fragment was replaced by an 8-mer *Xho* I linker; before the linker ligation, all termini were blunt-ended. The construction of pBATEM21 was described previously (Nagafuchi and Takeichi, 1988).

The nucleotide sequence of cDNAs encoding the mutant polypeptides was verified at least twice after the complete construction of each expression vector.

cDNA transfection

For the isolation of L cells stably transformed with plasmids encoding the mutant E-cadherin polypeptides, cells were co-transfected with a plasmid for neomycin resistance and selected with G418, as described previously (Nagafuchi *et al.,* 1987). Cells were grown in Dulbecco's modified Eagle's MEM (DMEM) supplemented with 10% fetal bovine serum.

Immunoblot analysis

Samples were separated by SDS-PAGE using 7.5% polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose sheets. Nitrocellulose transfers were incubated with a polyclonal rabbit antiserum (Nagafuchi *et al.,* 1987) to E-cadherin, followed by incubation with ^{125}I-labeled Protein A (Amersham). Radiolabeled electrophoretic bands were visualized by subjecting the transfers to autoradiography.

Trypsin treatment and aggregation of cells

Cells or cell membrane fractions were trypsinized in the presence of 2 mM Ca^{2+} (TC-treatment) or 1 mM EGTA (TE-treatment), as previously described (Yoshida *et al.,* 1982). In situ, cadherins are digested by TE-treatment but not by TC-treatment. When the membrane fraction of cells is treated, the extracellular domain of cadherins is cleaved to yield an 84 kDa fragment by TC-treatment and into smaller fragments by TE-treatment (Shirayoshi *et al.,* 1986).

For cell aggregation assays, 2×10^5 cells dispersed by TC-treatment were placed on each well of a Nunclon 24-well plate with 0.5 ml Hepes-buffered Mg^{2+}-free saline with 1 mM $CaCl_2$ (HMF) and allowed to aggregate for 30 min at 37°C, as described previously (Nagafuchi *et al.,* 1987). The extent of cell aggregation is represented by the index $(N_0 - N_{30})/N_0$ where N_{30} is the total particle number after a 30 min incubation period and N_0 is the total particle number at the initiation of incubation.

Detergent extraction of cells

Cultured cells were extracted with 2.5% NP40 in HMF, as described previously (Nagafuchi *et al.,* 1988). For SDS-PAGE, 4×10^7 cells were lysed in 1 ml of SDS lysis buffer.

Metabolic radiolabeling of cells and immunoprecipitation

For radiolabeling of cellular components, cells were incubated in methionine-free DMEM supplemented with 10% fetal calf serum with 100 μCi/ml of ^{35}S-labeled methionine for 8–15 h at 37°C. The labeled cells in a 5-cm culture dish were lysed in 1 ml of extraction buffer containing 0.5% NP40, 2 mM $CaCl_2$, 2 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM *p*-toluenesulfonyl-L-arginine methyl ester hydrochloride (TAME) in HMF, then centrifuged at 100,000 rpm for 30 min. To the supernatant, 50 μl of normal rabbit serum was added, and the solution was incubated with 100 μl of Affi-Gel Protein A beads (BioRad) for absorbing components which might nonspecifically bind to the beads. After removing the beads by centrifugation, the same incubation was repeated twice. Then, 5 μl of a rabbit antiserum raised against E-cadherin was added and incubated for 30 min. To this solution, 100 μl of the protein A beads were added and incubated for 20 min, and the beads were collected by centrifugation. The immune complexes were washed 6 times with the extraction buffer, then suspended in 50 μl of SDS-lysis buffer with 5% mercaptoethanol and boiled for 5 min. The released materials were subjected to SDS-PAGE using a 7.5% polyacrylamide gel. For autoradiography, signals were enhanced by the DMSO-PPO method (Bonner and Lasky, 1974).

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