

# FUNCTIONAL CORRELATION BETWEEN CELL ADHESIVE PROPERTIES AND SOME CELL SURFACE PROTEINS

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## ABSTRACT

The adhesive properties of Chinese hamster V79 cells were analyzed and characterized by various cell dissociation treatments. The comparisons of aggregatability among cells dissociated with EDTA, trypsin +  $\text{Ca}^{2+}$ , and trypsin + EDTA, revealed that these cells have two adhesion mechanisms, a  $\text{Ca}^{2+}$ -independent and a  $\text{Ca}^{2+}$ -dependent one. The former did not depend on temperature, whereas the latter occurred only at physiological temperatures. Both mechanisms were trypsin sensitive, but the  $\text{Ca}^{2+}$ -dependent one was protected by  $\text{Ca}^{2+}$  against trypsinization.

In morphological studies, the  $\text{Ca}^{2+}$ -independent adhesion appeared to be a simple agglutination or flocculation of cells, whereas the  $\text{Ca}^{2+}$ -dependent adhesion seemed to be more physiological, being accompanied by cell deformation resulting in the increase of contact area between adjacent cells.

Lactoperoxidase-catalyzed iodination of cell surface proteins revealed that several proteins are more intensely labeled in cells with  $\text{Ca}^{2+}$ -independent adhesiveness than in cells without that property. It was also found that a cell surface protein with a molecular weight of approximately 150,000 is present only in cells with  $\text{Ca}^{2+}$ -dependent adhesiveness. The iodination and trypsinization of this protein were protected by  $\text{Ca}^{2+}$ , suggesting its reactivity to  $\text{Ca}^{2+}$ . Possible mechanisms for each adhesion property are discussed, taking into account the correlation of these proteins with cell adhesiveness.

**KEY WORDS** cell adhesion · cell surface proteins ·  $\text{Ca}^{2+}$  · Chinese hamster cells · lactoperoxidase-catalyzed iodination

Cellular adhesiveness is one of the fundamental properties of cells constituting tissues in multicellular organisms. Basic studies on cell adhesion will provide important information for understanding control mechanisms in cell behavior and morphogenesis. Although there have been many studies of the adhesive properties of cells, the reports from different laboratories are often conflicting.

For example, temperature independence of cell aggregation was found by several workers (3, 16), whereas other authors obtained the opposite results (7, 17, 18, 28, 31, 34). Divalent cation dependence of cell adhesion has often been reported (1, 2, 11, 29, 31, 34), whereas recent studies revealed that some cell types do not require divalent cations for aggregation under some conditions (4, 8, 32). Such apparently conflicting results could be due to a difference in adhesive properties among different cell types and/or to the existence of multiple cell adhesion mechanisms which could

be detected differentially depending on experimental conditions. In the present study, we have compared the adhesiveness of Chinese hamster V79 cells suspended from monolayer cultures by a variety of methods. Our results show that this single cell type can display different adhesion mechanisms depending on the method of preparation of the cell suspension. The molecules that are possibly related to each mechanism were explored, using lactoperoxidase-catalyzed iodination of cell surface proteins.

## MATERIALS AND METHODS

### Cell

The Chinese hamster V79 lung cell line (9, 27) was used in this study. Cells were grown on plastic tissue culture plates (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in Eagle's Minimal Essential Medium (MEM) supplemented with 15% fetal calf serum, Grand Island Biological Co., Grand Island, N.Y.), 0.2% lactalbumin hydrolysate (GIBCO), and 58.5 mg/l L-glutamine.

### Preparation of Single Cells

Cell monolayers that had just attained a confluent state were used for preparing single cells. Such cultures were usually obtained by inoculating  $1.2\text{--}1.4 \times 10^4$  cells/cm<sup>2</sup> culture dish area, and by incubating for 2 days at 37°C. Overcrowded cultures were avoided because they failed to give well-dispersed single cells. Three different methods were used to dissociate the cell monolayers: the resulting cell suspensions are designated E-, TC-, or TE-cells, depending on the method of dissociation. (a) E-cells: A cell monolayer on a 14-cm diameter culture dish was rinsed three times with Puck's Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free saline (CMF, reference 25), and incubated with 10 ml of 1 mM EDTA in 10 mM HEPES-buffered saline (HCMF; 8.0 g of NaCl, 0.4 g of KCl, 0.09 g of Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 1 g of glucose, 2.38 g of HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid), and 4.8 ml of 1 N NaOH in 1,000 ml of H<sub>2</sub>O, pH 7.4) for 15 min at 37°C. (b) TC-cells: A cell monolayer on the same-size plate was rinsed three times with 1 mM CaCl<sub>2</sub> in CMF and incubated with 10 ml of 0.01% crystalline trypsin (type I, Sigma Chemical Co., St. Louis, Mo.) in HCMF containing 0.1 mM CaCl<sub>2</sub> for 15 min at 37°C. (c) TE-cells: EDTA in method 1 was replaced by 0.01% crystalline trypsin in HCMF containing 1 mM EDTA. All these incubations were done on a gyratory shaker (model G-76, New Brunswick Scientific Co., Inc., New Brunswick, N.J.) at 100 rpm. Cells detached from the plate were centrifuged and resuspended in 5 ml of CMF. This CMF was supplemented with 0.01% soybean trypsin inhibitor (type I-S, Sigma Chemical) in cases where cells had been trypsinized. Cells were washed two more times with CMF and finally suspended in the medium to

be used for the experiment. The above-described methods were modified, when necessary, as described in the text.

### Measurement of Cell Aggregation

Plastic wells (2.8 cm x 1.5 cm) were used for the assay of cell aggregation as previously described (31, 32).  $1 \times 10^6$  cells suspended in 3 ml of HCMF were put into each well, which had been previously coated with bovine serum albumin (BSA; Armour Fraction V, Armour Pharmaceutical Co., Chicago, Ill.) to prevent attachment of cells to the plastic (29). After addition of a reagent the effect of which was to be tested, cells were incubated at 37°C on a gyratory shaker at 80 rpm. The aggregation was stopped with a gentle swirling of the dish and subsequent addition of 2 ml of 5% glutaraldehyde in HCMF. It was previously determined that this fixation procedure does not cause any artificial aggregation or dissociation of preformed aggregates (31). To measure cell aggregation, the total particle number in cell suspension was counted with a Coulter counter (model Z<sub>B</sub>; Coulter Electronics Inc., Hialeah, Fla.) with 100- $\mu$ m aperture. The extent of aggregation was represented by the ratio of the total particle number at time *t* of incubation (*N<sub>t</sub>*) to the initial particle number (*N<sub>0</sub>*), the latter being identical to the total number of cells added to the medium. The validity of this method has been previously established (6, 33).

### Iodination of Cells

Dissociated cells were suspended in cold HCMF with 10 mM glucose at a cell concentration of  $3 \times 10^6$  cells/ml. <sup>125</sup>I-Na (5 mCi/0.1 ml, carrier free, New England Nuclear, Boston, Mass.) was added to a final concentration of 200 or 400  $\mu$ Ci/ml, followed by lactoperoxidase (B grade, Calbiochem, San Diego, Calif.) and glucose oxidase (type V, Sigma Chemical) to final concentrations of 20  $\mu$ g/ml and 0.2 U/ml, respectively. The lactoperoxidase was previously dialyzed against HCMF to remove contaminating Ca<sup>2+</sup>. The reaction mixture was incubated at 5°C for 7 min with occasional swirling, and the cells were sedimented and washed four to five times with Hanks' saline solution. Cell viability and aggregability were not affected by iodination.

### Sodium Dodecyl Sulfate-Polyacrylamide

#### Gel Electrophoresis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was done by the method of Laemmli (15) with modifications. The separation gel solution consisted of 7.5% acrylamide, 0.375 M Tris-HCl (pH 8.8), 0.2% *N,N'*-methylene-bis-acrylamide, and 0.1% SDS. The gel was polymerized in a slab (1 mm x 17 cm) by the addition of 0.05% *N,N,N',N'*-tetramethylethylenediamine (TEMED) and 0.05% ammonium persulfate (final concentrations). The stacking gel, which contains 3.2% acrylamide, 0.125 M Tris-HCl (pH 6.8), 0.36% *N,N'*-methylene-bis-acrylamide, and

0.1% SDS, was polymerized with 0.1% TEMED and 0.16% riboflavin. The electrode buffer contained 6 g of Tris, 28.8 g of glycine and 1 g of SDS in 1,000 ml. Cells were dissolved in 0.125 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 2 mM phenylmethylsulfonylfluoride, and 0.001% bromphenol blue (sample buffer). After addition of 5–10% 2-mercaptoethanol, the samples were incubated in boiling water for 2 min. Electrophoresis was carried out at 11 mA until the bromphenol blue marker reached a distance of 13 cm from the top of the separation gel. The gels were stained with 0.25% Coomassie Blue in 25% 2-propanol and 10% acetic acid. After destaining with 5% ethanol and 10% acetic acid, gels were dried under vacuum and autoradiographed with X-ray film (RP/R54 Eastman Kodak Co., Rochester, N.Y.).

### Other Reagents

Other reagents used were obtained from the following sources: cycloheximide, dithiothreitol,  $\beta$ -galactosidase, catalase, and ovalbumin from Sigma Chemical, colcemid from GIBCO, tetracaine from Schwarz/Mann Div., Becton, Dickson & Co. (Orangeburg, N.Y.), cytochalasin B from Imperial Chemical Industries Ltd. (Cheshire, England), and neuraminidase from Calbiochem. A23187 was a gift from Eli Lilly and Co. (Indianapolis, Indiana). Myosin was extracted from mouse leg by the method of Perry (21).

## RESULTS

### Aggregation of Cells after Different Treatments for Dissociation

The reaggregative properties of cells dissociated with 1 mM EDTA, 0.01% trypsin + 0.1 mM  $\text{CaCl}_2$ , or 0.01% trypsin + 1 mM EDTA

were compared. Cells dissociated with 1 mM EDTA (E-cells) aggregated rapidly (Fig. 1a). This aggregation was enhanced by the addition of 1 mM  $\text{Ca}^{2+}$ , but occurred even in the absence of divalent cations. Cells dissociated with 0.01% trypsin + 0.1 mM  $\text{Ca}^{2+}$  (TC-cells) did not aggregate in the absence of divalent cations, but rapidly aggregated in the presence of  $\text{Ca}^{2+}$  (Fig. 1b). Cells dissociated with 0.01% trypsin + 1 mM EDTA completely lost their aggregability and exhibited no  $\text{Ca}^{2+}$  effect (Fig. 1c).

The effect of various concentrations (0.01–10 mM) of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  on the aggregation of these three types of cells was studied. For E- and TC-cells,  $\text{Ca}^{2+}$  was maximally effective at 1 mM. TE-cells did not aggregate at any concentration of  $\text{Ca}^{2+}$ .  $\text{Mg}^{2+}$  enhanced the aggregation of all three cell types, but to a much lesser degree than did  $\text{Ca}^{2+}$  for E- or TC-cells, suggesting different specificities for the two ions.

E-cells were treated with 0.01% trypsin (with or without  $\text{Ca}^{2+}$ ) to determine whether such cells had an identical aggregation pathway to TC-cells. E-cells treated with trypsin +  $\text{Ca}^{2+}$  retained their ability to aggregate in the presence of  $\text{Ca}^{2+}$ , but lost the ability to aggregate in the absence of  $\text{Ca}^{2+}$  (Table I). This property is exactly the same as that of TC-cells. E-cells treated with trypsin without  $\text{Ca}^{2+}$  completely lost their aggregability, exactly like TE-cells. These results show that E-cells possess the same  $\text{Ca}^{2+}$ -dependent adhesive property found in TC-cells.

The effect of temperature on the aggregation of E- and TC-cells was studied (Fig. 2). E-cells

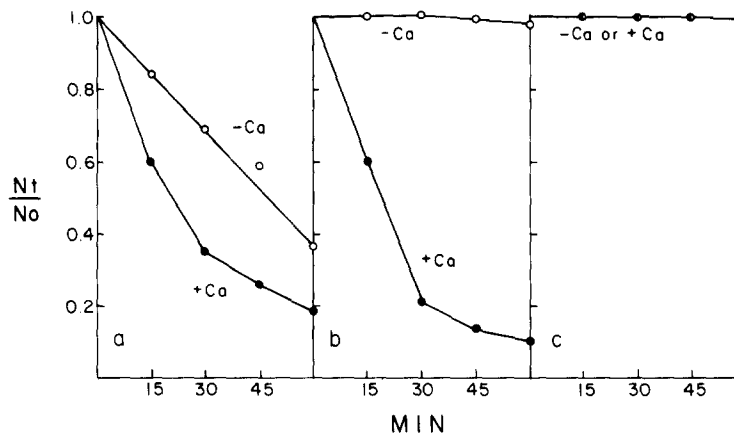


FIGURE 1 Aggregation of cells dissociated with 1 mM EDTA (a), 0.01% trypsin + 0.01 mM  $\text{Ca}^{2+}$  (b), and 0.01% trypsin + 1 mM EDTA (c). Medium for aggregation contains 1 mM  $\text{Ca}^{2+}$  (●—●), or no divalent cation (○—○).

TABLE I  
Aggregation of Trypsinized E-Cells

Trypsinization*	Aggregation (Nt/No)‡	
	In 1 mM Ca <sup>2+</sup>	Without Ca <sup>2+</sup>
With 1 mM Ca <sup>2+</sup>	0.39	0.99
Without Ca <sup>2+</sup>	0.97	0.96

\* E-cells ( $1 \times 10^7$  cells) washed once with HCMF or 1 mM Ca<sup>2+</sup> in HCMF were incubated in 2 ml of 0.01% trypsin in the absence or presence of 1 mM Ca<sup>2+</sup>, respectively, at 37°C for 10 min.

‡ Aggregation was assayed at 30 min of incubation.

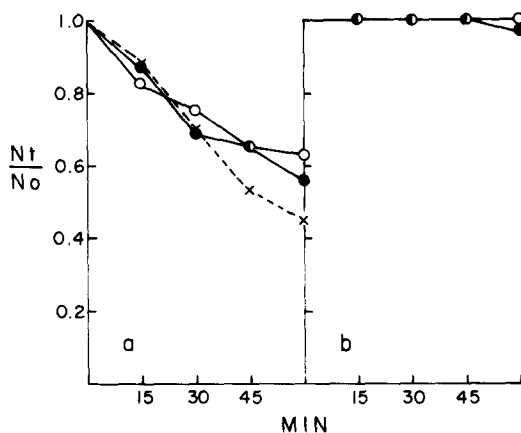


FIGURE 2 Effect of temperature on cell aggregation. (a) E-cells in the presence (●—●) and absence (○—○) of 1 mM Ca<sup>2+</sup> at 2°C, and in the absence of Ca<sup>2+</sup> at 37°C (×—×). (b) TC-cells in the presence (●—●) and absence (○—○) of 1 mM Ca<sup>2+</sup> at 2°C.

aggregated at a low temperature (2°C); however, Ca<sup>2+</sup> did not promote this aggregation. Interestingly, the rate of aggregation of E-cells at the low temperature was almost the same as that at 37°C in Ca<sup>2+</sup>-free medium (Fig. 2a). The aggregation of TC-cells was totally inhibited at low temperature (Fig. 2b).

#### Ca<sup>2+</sup>-Protective Effect on Cell Adhesiveness against Trypsinization

The effect of various cations as substitutes for Ca<sup>2+</sup> to obtain the same aggregative properties of TC-cells was examined. In these experiments, the cell monolayer was rinsed with CMF supplemented with the ion to be tested and was subsequently trypsinized (15 min at 37°C) in the presence of that ion. Cell aggregation was then assayed in the presence of 1 mM Ca<sup>2+</sup> after 30 min of incubation. Mg<sup>2+</sup> (1–10 mM) and Mn<sup>2+</sup> (0.1

mM) could not substitute for Ca<sup>2+</sup>. Aggregative properties of cells trypsinized in the presence of these ions were identical to those of TE-cells.

To determine whether or not the adhesion sites on the cell surface are directly protected by Ca<sup>2+</sup> from trypsin digestion, the aggregation of cells in the presence of trypsin was examined. TC-cells were suspended in HCMF at room temperature in the dishes to be used for the aggregation experiment. 1 mM Ca<sup>2+</sup> was then added to the cell suspension, followed by trypsin (0.01% final concentration) 1 or 5 min later. The cells were brought to 37°C on a gyratory shaker to examine their aggregatability. The results, shown in Table II, revealed that aggregation was totally unaffected by the presence of trypsin if Ca<sup>2+</sup> was added 5 min before addition of the enzyme. Addition of trypsin as early as 1 min after Ca<sup>2+</sup> resulted in small numbers of aggregating cells. As expected, when trypsin was added to the cells before the addition of Ca<sup>2+</sup>, no cell aggregation occurred. These experiments show that Ca<sup>2+</sup> directly protects the adhesive property of cells against trypsinization.

#### Effect of Preincubation of TC-cells in Ca<sup>2+</sup>

The question of whether TC-cells acquire adhesive capacity irreversibly during incubation with Ca<sup>2+</sup> was tested. TC-cells were incubated with 1 mM Ca<sup>2+</sup> at 37°C for 60 min on a gyratory shaker at high speed (180 rpm) preventing their aggregation. The cells were collected, rinsed with

TABLE II  
Effect of Trypsin during Cell Aggregation

Trypsin treatment*	Aggregation (Nt/No)‡
Trypsin added	
5 min before the addition of Ca <sup>2+</sup>	0.98
1 min before the addition of Ca <sup>2+</sup>	1.03
1 min after the addition of Ca <sup>2+</sup>	0.77
5 min after the addition of Ca <sup>2+</sup>	0.34
No trypsin§	0.32

\* Trypsin (0.01%) and Ca<sup>2+</sup> (1 mM) were added from 10- and 100-fold concentrated stock solutions, respectively.

‡ Aggregation was assayed at 30 min after the final addition of trypsin or Ca<sup>2+</sup>.

§ Control contains 1 mM Ca<sup>2+</sup>.

CMF, and reincubated with or without 1 mM  $\text{Ca}^{2+}$  to assay their aggregability. The result showed that cells preincubated with  $\text{Ca}^{2+}$  still require  $\text{Ca}^{2+}$  for aggregation in the medium. TC-cells, therefore, need the continuous presence of  $\text{Ca}^{2+}$  to display their adherent property.

#### Recovery of $\text{Ca}^{2+}$ -Dependent Adhesiveness in TE-Cells

The  $\text{Ca}^{2+}$ -dependent adhesiveness which is absent in TE-cells should be recovered metabolically after protein synthesis if the responsible sites were digested by trypsin. This was confirmed as follows. TE-cells were plated with normal cell culture medium containing serum (see Materials and Methods) onto Falcon plastic tissue culture dishes (Falcon Plastics). After appropriate incubation periods, they were harvested by the method for making TC-cells, and their aggregability was assayed. Control plates contained 100  $\mu\text{g}/\text{ml}$  cycloheximide to inhibit protein synthesis. Cycloheximide tended to inhibit cell attachment to the plate and also cell spreading on it; only cells attached on the plate were used for this experiment. Fig. 3 shows that cells start to recover their  $\text{Ca}^{2+}$ -dependent adhesiveness between about 1 and 2.5 h of incubation and acquire considerable adhesive ability by 6 h. Cycloheximide completely inhibited this recovery.

#### Effect of Other Reagents on Aggregation of TC-Cells

As summarized in Table III, colcemid, tetracaine, neuraminidase, or the  $\text{Ca}^{2+}$ -ionophore

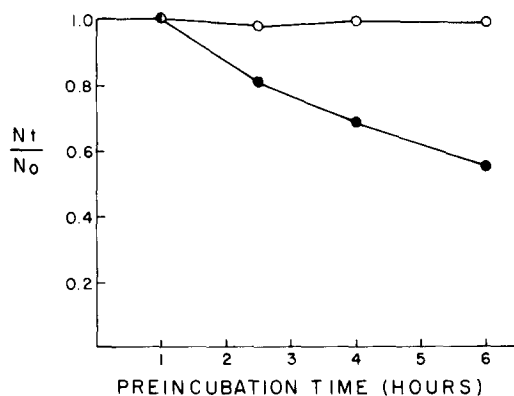


FIGURE 3 Recovery of  $\text{Ca}^{2+}$ -dependent adhesiveness in TE-cells. Cells were cultured for the indicated periods with (○—○) or without (●—●) 100  $\mu\text{g}/\text{ml}$  cycloheximide. The aggregation was assayed at 30 min of incubation.

TABLE III  
Effect of Miscellaneous Reagents on TC-Cell Aggregation

Reagents	Aggregation (Nt/No)*
A Control	
1 mM $\text{Ca}^{2+}$	0.22
0.1 mM $\text{Ca}^{2+}$	0.99
0.01 mM $\text{Ca}^{2+}$	1.00
B 1 $\mu\text{M}$ A23187 + 1 mM $\text{Ca}^{2+}$	0.21
+ 0.1 mM $\text{Ca}^{2+}$	1.00
+ 0.01 mM $\text{Ca}^{2+}$	1.01
C ‡ 100 U/ml neuraminidase	0.23 (0.97)
D § 1 mM tetracaine	0.19
10 $\mu\text{g}/\text{ml}$ colcemid	0.22
10 $\mu\text{g}/\text{ml}$ cytochalasin B	0.38
100 $\mu\text{g}/\text{ml}$ cycloheximide	0.32
E    10 mM dithiothreitol	0.94

\* Aggregation was assayed at 30 min of incubation in the presence of the indicated concentrations of  $\text{Ca}^{2+}$  in A and B, and in the presence of 1 mM  $\text{Ca}^{2+}$  in C, D, and E. ‡ Cells pretreated with the enzyme in HCMF for 15 min at 37°C were rinsed with CMF and allowed to aggregate. The value in parentheses shows the aggregability in  $\text{Ca}^{2+}$ -free medium.

§ Cells were preincubated with each reagent in HCMF for 30 min at room temperature. Then  $\text{Ca}^{2+}$  was added, and the preparation was brought to 37°C.

|| Cells were preincubated with dithiothreitol in HCMF at 37°C for 30 min, washed, and allowed to aggregate.

A23187 had no effect on TC-cell aggregation. Cytochalasin B and cycloheximide inhibited aggregation only slightly. TC-cells preincubated with 10 mM dithiothreitol in HCMF at 37°C for 30 min lost their aggregability without a significant reduction in cell viability. This result suggests that sulfhydryl groups may be important in the function of adhesion sites.

#### Morphological Studies of Cell Aggregation

Cell aggregates formed after a 60-min incubation at 37°C were subjected to further morphological studies. E-cells in the presence of  $\text{Ca}^{2+}$  tightly adhered to one another with large contact areas (Fig. 4a), suggesting that the deformation of cells occurred to increase the contact area between the apposed cells after the aggregation. TC-cells in the presence of  $\text{Ca}^{2+}$  also adhered to one another over large contact areas (Fig. 4c). A peculiar feature of TC-cell aggregates was that those cells were associated with one another often as rodlike one-dimensional chains of cells, which branched in several directions.

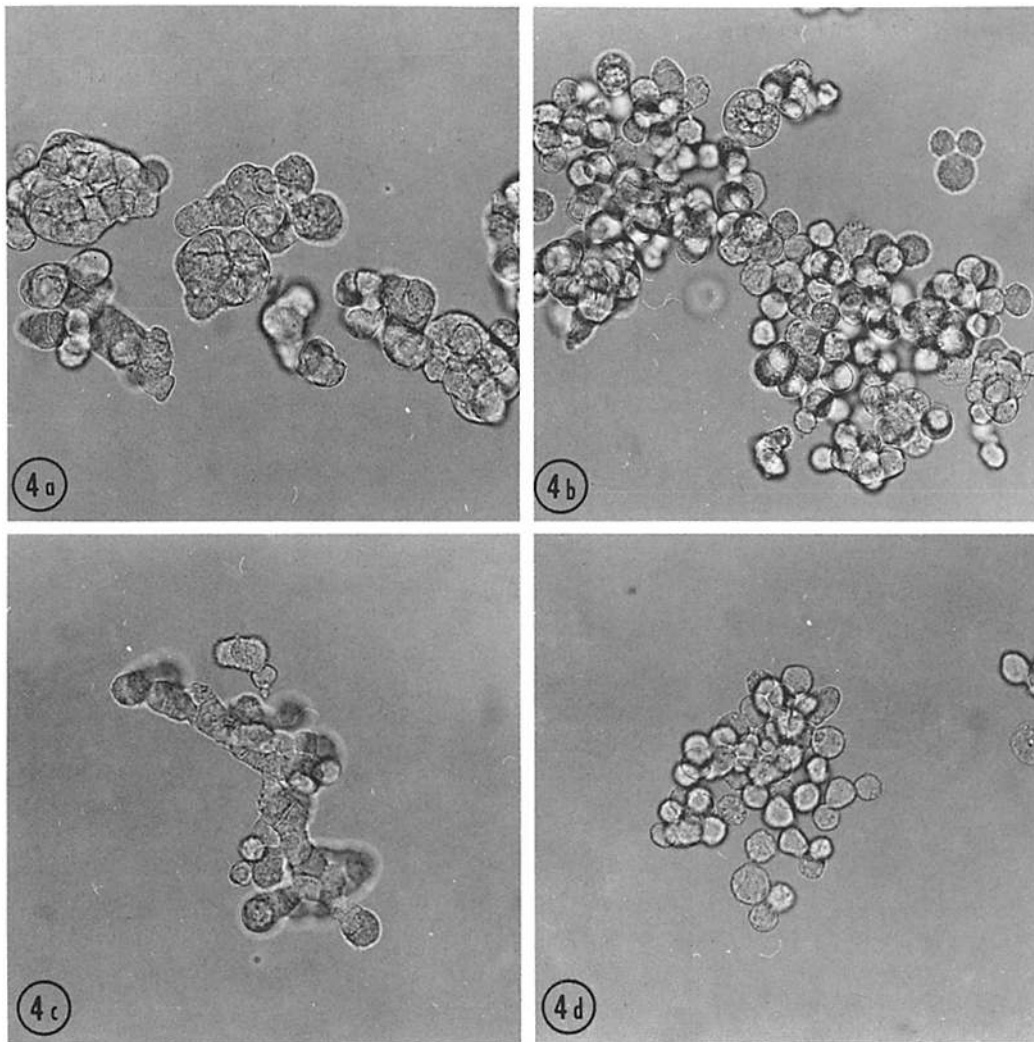


FIGURE 4 Photomicrographs of cell aggregates. E-cells in 1 mM  $\text{Ca}^{2+}$  (a), in no divalent cations (b), and in 1 mM  $\text{Mg}^{2+}$  (d); TC-cells in 1 mM  $\text{Ca}^{2+}$  (c). Cells were incubated for 60 min at 37°C.  $\times 360$ .

Aggregates of E-cells in  $\text{Ca}^{2+}$ -free medium showed a different morphology. Individual cells within an aggregate remained round like unaggregated cells (Fig. 4b).  $\text{Mg}^{2+}$  had no effect on the morphology of E-cell aggregates (Fig. 4d).

#### *Correlations of Cell Adhesiveness to Cell Surface Proteins*

To further characterize the cell surface components associated with the cell adhesion properties observed above, the lactoperoxidase-catalyzed iodination of intact cells (12, 22, 23) was examined, and the iodinated protein species were compared among E-, TC-, and TE-cells by SDS-

polyacrylamide gel electrophoresis. Those cells freshly prepared were iodinated in HCMF at 5°C. Fig. 5 (a, b, and c) shows autoradiographs of the SDS gel electrophoresis patterns obtained from the whole cell proteins extracted from iodinated E-, TC-, and TE-cells. A number of radioactive bands appeared in all three types of cells. Several bands, particularly two components whose molecular weight (mol wt) is approximately 60,000, were, however, apparently more intensely labeled in E-cells than in trypsinized cells. When the radioactive bands of TC- (Fig. 5b) and TE-cells (Fig. 5c) were compared, one clear difference was observed. A radioactive protein with a molecular weight of approximately

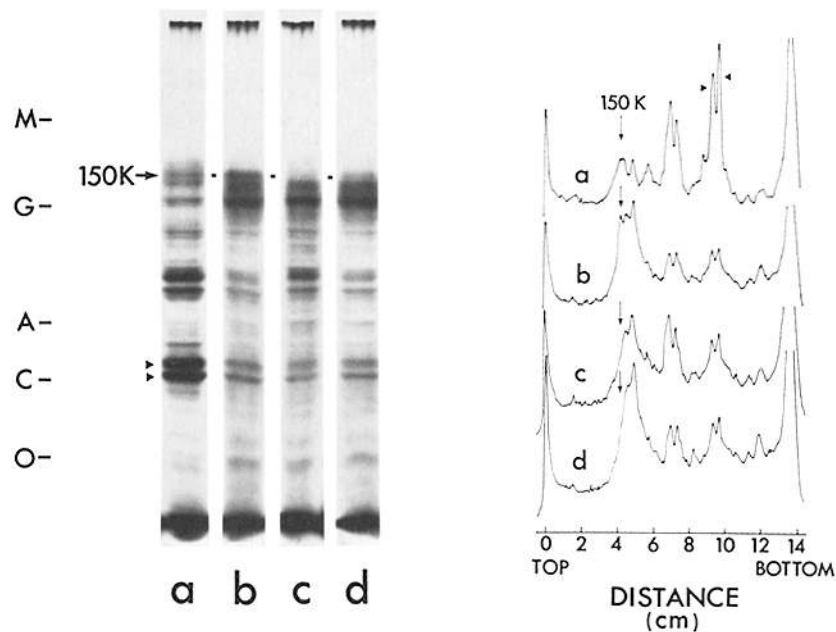


FIGURE 5 Autoradiographs of SDS-polyacrylamide gels after electrophoresis of proteins from iodinated cells (left) and their densitometer traces (right). E-cells (*a*), TC-cells (*b*), and TE-cells (*c*) iodinated in the absence of divalent cations; TC-cells iodinated in the presence of 1 mM  $\text{Ca}^{2+}$  (*d*). In the latter case, TC-cells were prewashed before iodination with 1 mM  $\text{Ca}^{2+}$  in HCMF.  $1.5 \times 10^6$  cells were dissolved in 100  $\mu\text{l}$  of SDS-sample buffer, and 30  $\mu\text{l}$  of this extract was applied to electrophoresis for each cell type. Two components with a mol wt of approximately 60,000 are shown by closed triangles ( $\blacktriangle$ ). The positions of the molecular weight markers are indicated: myosin (210,000), *M*;  $\beta$ -galactosidase (130,000), *G*; BSA (68,000), *A*; catalase (57,500), *C*; and ovalbumin (43,500), *O*.

150,000 (abbreviated 150K protein) appeared predominantly in TC-cells. No other differences were observed between TC- and TE-cells, even when 15% polyacrylamide gels were used to separate lower molecular weight proteins. Interestingly, the iodination of the 150K protein was remarkably suppressed when the iodination medium contained 1 mM  $\text{Ca}^{2+}$  (Fig. 5 *d*), whereas 1 mM  $\text{Mg}^{2+}$  had no effect on the iodination pattern. Iodination of other components was not affected by  $\text{Ca}^{2+}$ . No differences in the iodination pattern for TC- and TE-cells were observed after preincubation of the cells in the presence of 1 mM  $\text{Ca}^{2+}$  (30 min at 37°C), except for the 150K protein.

A correlation of the recovery of  $\text{Ca}^{2+}$ -dependent adhesiveness in TE-cells with the appearance of the 150K protein species was suggested by the following iodination experiments. "Recovered" TE-cells were obtained as described in the previous section, and subjected to  $^{125}\text{I}$ -labeling. As shown in Fig. 6, the 150K protein appeared in TE-cells after their recovery (6 h) in normal

culture medium, but did not appear in the presence of cycloheximide. Even though the 150K protein band shown here was not so intensely radiolabeled as that found on freshly prepared TC-cells, it is noteworthy that the recovery of  $\text{Ca}^{2+}$ -dependent adhesiveness in TE-cells was also not complete.

#### DISCUSSION

The present studies suggest that V79 cells have at least two mechanisms for cell-to-cell adhesions: a  $\text{Ca}^{2+}$ -independent and a  $\text{Ca}^{2+}$ -dependent one.  $\text{Ca}^{2+}$ -independent adhesion does not depend on temperature and is not accompanied by morphological change of cells after aggregation. In contrast,  $\text{Ca}^{2+}$ -dependent adhesion requires physiological temperatures, and cells actively spread over one another after aggregation. Both mechanisms are trypsin-sensitive, but  $\text{Ca}^{2+}$ -dependent adhesiveness was protected by  $\text{Ca}^{2+}$  against trypsinization.  $\text{Ca}^{2+}$ -dependent adhesiveness was not made  $\text{Ca}^{2+}$ -independent by preincubation of cells with  $\text{Ca}^{2+}$ . These observations demonstrate that

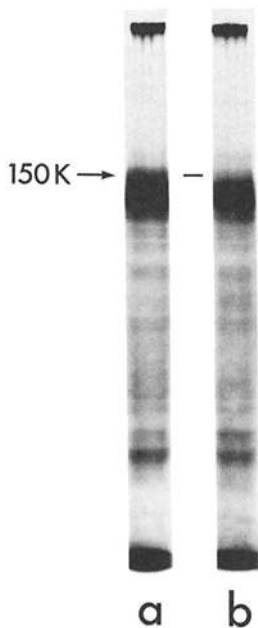


FIGURE 6 Autoradiographs of SDS-polyacrylamide gels after electrophoresis of proteins from iodinated TE-cells. Preincubated before iodination for 6 h in the absence (a) and the presence (b) of 100  $\mu\text{g/ml}$  cycloheximide as described in the text.

the two adhesion mechanisms are totally independent.

Recent studies have shown that some cell types do not necessarily require divalent cations for mutual adhesion under some conditions (4, 8, 32), suggesting the wider generality of the  $\text{Ca}^{2+}$ -independent mechanism shown here. Our findings are also consistent with the results of Curtis and Greaves (3) that EDTA-dissociated cells (chick embryonic) are able to aggregate at low temperature. The temperature independence of the  $\text{Ca}^{2+}$ -independent adhesion suggests that this adhesion does not involve any active cellular process but some direct chemical or physicochemical interactions among cell surface components. Results of microscopic observations are consistent with this idea, because cells exhibited almost no morphological changes in aggregation, as seen in the agglutination or flocculation of nonliving particles.

Inasmuch as  $\text{Ca}^{2+}$ -independent adhesiveness of E-cells was abolished by the trypsin treatment, some proteins should be involved in the adhesion process. Iodination of intact cells demonstrated that several proteins are more intensely labeled in E-cells than in TC-cells, suggesting that one of

those could be an important component for the adhesion. We have recently studied the adhesion of artificially generated phospholipid membrane vesicles to EDTA-dissociated cells (20). It was shown that the adhesion of such lipid vesicles is mediated by the 60,000 mol wt cell surface proteins, which are found predominantly in E-cells (Fig. 5a). Adhesion of the lipid vesicles to the cell surface does not require divalent cations and takes place at low temperature. These phenomena suggest that the 60,000 mol wt proteins may play some role in  $\text{Ca}^{2+}$ -independent adhesion, perhaps involving lipid-protein interaction between two cell surfaces (20).

$\text{Ca}^{2+}$ -dependent adhesion, which occurs only at a physiological temperature, might be related to other cellular processes such as enzymatic reactions, changes in fluidity of the plasma membrane, cellular motility, or contractile activity. Morphological studies show that E-cells or TC-cells increase contact areas by spreading over onto adjacent cells during aggregation in the presence of  $\text{Ca}^{2+}$ , but not in the absence of  $\text{Ca}^{2+}$ . This phenomenon suggests the possibility that the primary role of  $\text{Ca}^{2+}$  is to enhance the activity of pseudopodia or other cell motility machineries and to indirectly promote cell-cell adhesions by increasing the ability of cells to actively hold neighboring cells. This possibility, however, is unlikely for the following reasons. We observed that TC- and TE-cells exhibited a similar spreading activity with pseudopodia on a Falcon plastic culture dish (unpublished data), suggesting that the motility of those two cell types is similar. Furthermore, the drugs cytochalasin B, colcemid, and tetracaine, which are known to affect cellular motility, hardly inhibited the TC-cell aggregation. It thus seems that the motility of cells is not importantly relevant to the role of  $\text{Ca}^{2+}$  in rendering cells adhesive. Probably, the observed increase of contact area among adhered cells in TC- and E-cell aggregates in  $\text{Ca}^{2+}$  rather represents their high adhesiveness to one another, inasmuch as cells have the property of spontaneously spreading over the very adhesive substrate, as seen in cells plated on clean glass or plastic.

Studies of the mobility of membrane antigens or lectin receptors have revealed that these cell surface components can freely move laterally in the plasma membrane, the movement being temperature-dependent (5). It is reasonable to assume that the temperature dependence of  $\text{Ca}^{2+}$ -



dependent adhesion is associated with the mobility of adhesion sites in plasma membranes. Presumably, the components necessary for adhesion on one cell surface must move laterally to be coupled with complementary components on the surfaces of adjacent cells as discussed by several authors (8, 19, 28). In particular, if the adhesion requires a special arrangement of those components, such as cluster or patch formation, physiological temperatures would be absolutely necessary for that process, as shown in the case of lectin receptors or membrane antigens (5, 19). The fact that drugs known to interfere with the movement of such membrane components (cytochalasin B, colcemid, and tetracaine) hardly affected the  $\text{Ca}^{2+}$ -dependent adhesion does not seem to contradict the idea mentioned above, because these drugs do not necessarily inhibit the movement of membrane components (19). Therefore, it can be proposed that  $\text{Ca}^{2+}$  is required for maintaining a particular arrangement of junctional components or for activating these components to make them functional. The one-dimensional arrangement of cells in TC-cell aggregates can be elucidated by assuming that the adhesion sites are sparsely located on the cell surface, being regulated by the lateral movement of the components in the plasma membrane. The disordering of a unique arrangement of membrane particles in the tight junction by the removal of  $\text{Ca}^{2+}$  from the medium has been recently reported (10).

Iodination of intact cells revealed that cells with the  $\text{Ca}^{2+}$ -dependent adhesiveness possess one radiolabeled component (150K protein), which was not found in nonadhesive TE-cells. Because it has been known that in the lactoperoxidase-catalyzed iodination system only proteins located on the cell surface are labeled under proper conditions (13, 14, 30), this 150K protein is probably a cell surface component. This idea is consistent with the fact that the 150K protein can be removed from cells by trypsin. The origin of this protein appeared to be cell derived. When TE-cells were cultured in media containing serum with or without cycloheximide, the 150K protein was recovered only in the absence of this drug. If the 150K protein is serum or some other exogenous component adsorbed on the cell surface, both cells should have equally contained this protein, but this was not found. However, we can not rule out one possibility that the 150K protein is an exogenous material and that the

protein synthesis inhibitor might have prevented the recovery of some cellular components which were required for the adsorption of this protein to the cell surface.

An interesting property of the 150K protein is its ability to react to  $\text{Ca}^{2+}$ ; this protein was protected by  $\text{Ca}^{2+}$  against iodination as well as against trypsinization. This suggests that the 150K protein undergoes conformational or positional changes in the plasma membrane by reacting to  $\text{Ca}^{2+}$ , which lead to the modification of its susceptibility to both lactoperoxidase and trypsin. Thus, it can be assumed that this protein is involved in the  $\text{Ca}^{2+}$ -dependent adhesion mechanism through such a property to react to  $\text{Ca}^{2+}$ . This idea is supported by the observation that the 150K protein is the only component that distinguishes between TC- and TE-cells, as far as studied by the present technique, and is also consistent with the proposed role of  $\text{Ca}^{2+}$  in the adhesion as discussed above. Rutishauser et al. (26) recently described a cell surface protein of a molecular weight of 150,000 which seems to be related to the adhesion activity of neural retina cells. Inasmuch as the adhesiveness of neural retina cells was protected by  $\text{Ca}^{2+}$  against trypsinization (28) and because their aggregation depended on the presence of  $\text{Ca}^{2+}$  (2, 31), both V79 and neural retina cells might have similar mechanisms for cell adhesion. Further studies, however, are necessary to confirm the importance of the 150K protein and to test the possibility of the existence of other components important for adhesion by using different techniques.

In the present study, evidence has been presented showing multiple adhesion mechanisms in one cell type. Presumably, each mechanism plays a different role in cell adhesion and contact. For example, the  $\text{Ca}^{2+}$ -independent property may be important in preserving the cell's general adhesiveness, whereas the  $\text{Ca}^{2+}$ -dependent adhesion sites might play a more dynamic role in cell behavior, such as regulation of contact inhibition of cell overlapping or of cell fusion which requires  $\text{Ca}^{2+}$  (24).

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