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Soluble 80-kd Fragment of Cell-CAM 120/80 Disrupts Cell-Cell Adhesion

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Calcium-dependent cell adhesion molecules (CAMs) mediate intercellular adhesion in epithelial cells and in preimplantation mammalian embryos. One of these molecules, cell-CAM 120/80, is found on cells as a 120-kd membrane glycoprotein and as a soluble 80-kd species in conditioned culture medium [Damsky et al: Cell 34:455, 1983]. We have purified to homogeneity the soluble 80-kd fragment of cell-CAM 120/80 by using monoclonal antibody affinity chromatography. We have shown that the purified molecule can disrupt cell-cell adhesion in cultured epithelial cells, thus indicating that it is directly involved in the adhesive process. In addition, we have further characterized both the 120-kd cell-associated molecule and its 80-kd fragment, including N-terminal sequence analysis.

Key words: calcium-dependent cell adhesion, epithelial cells, cell-CAM 120/80

Calcium-dependent epithelial cell adhesion molecules (CAMs) have been described in several systems. These include E-cadherin [2], uvomorulin [3,4], L-CAM [5–7], and cell-CAM 120/80 [1]. Cell-CAM 120/80 is unique in that it is the only one of these CAMs described in a human system. Antibodies raised against these CAMs disrupt cell-cell adhesion, indicating their importance in the adhesive process. Antibodies have also been used in each system to establish that these molecules are developmentally regulated and restricted to epithelial cells in the adult [1,8–11].

It has been demonstrated that E-cadherin and uvomorulin, both identified in mouse embryonal carcinoma cells, are the same molecule [12]. L-CAM from embryonic chicken liver [5] and cell-CAM 120/80 from cultured human tumor cells [1] are similar to one another and to uvomorulin or E-cadherin in molecular weight, calcium protection, and pattern of tissue distribution in developing and mature organisms.

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Their key role in the adhesive interactions during early development is demonstrated by the fact that antibodies against this class of calcium-dependent CAMs disrupt compaction of eight-cell embryos [10,13] and segregation of primitive endoderm from inner cell mass [8].

Cell-CAM 120/80 is found at cell-cell boundaries of human epithelial cells as a 120-kd glycoprotein (gp120). When MCF-7 human mammary carcinoma cells are maintained in serum-free culture medium, an 80-kd soluble fragment (gp80) is shed into the medium. A stable gp80 fragment can also be artificially generated by trypsinizing cells in the presence of calcium [1]. Protection from trypsin degradation by calcium is a characteristic of calcium-dependent cell adhesion molecules as defined by M. Takeichi [14].

Despite the extensive information that implicates these CAMs as participants in cell-cell adhesion, no direct effect on adhesion by the proteins themselves has been demonstrated. All data, to date, rely on the ability of these molecules to neutralize the adhesion-disrupting effect of specific antibodies. In this manuscript, we demonstrate that a sufficient quantity of highly purified gp80 can itself disrupt cell-cell adhesion, showing that this molecule participates directly in adhesion. We also present biochemical characterization data including N-terminal sequence analysis of cell-CAM 120/80. In these studies, we show that cell-CAM 120/80 is very similar to uvomorulin and L-CAM and also point out interesting differences.

MATERIALS AND METHODS

Cell Culture

The MCF-7 human mammary carcinoma cell line used to produce the 80-kd fragment of cell-CAM 120/80 was maintained as described [15]. Serum-free conditioned medium (SFM) was prepared from the culture supernatant of these cells as described [1]. Mouse mammary tumor epithelial cells (MMTE), used for bioassays, were maintained as described [1]. JAR PR497 human gestational choriocarcinoma cells (JAR) [16,17], a gift from P. Andrews, Wistar Institute, were maintained as described [1]. These were used as a source of gp120, as it is more stable during extraction in this line than in MCF-7 cells. P_3-X63Ag8 cells [18,19] were grown in Dulbecco's Modified Eagle Medium (DMEM, Flow Labs, Inglewood, CA) supplemented with 10% fetal calf serum (FCS). Hybridomas were grown in HY medium (Flow Labs) supplemented with 20% FCS.

Protease Inhibitors

Aprotinin, leupeptin, soybean trypsin inhibitor, and E amino caproic acid (all from Sigma, St. Louis, MO) were dissolved in H_2O at 20 mg/ml and stored at −20°C. Antipain (Sigma) was dissolved in H_2O at 40 mg/ml and stored at −20°C. Pepstatin A and chymostatin (Sigma) were dissolved in dimethylsulfoxide at 20 mg/ml and stored at −20°C. Protease inhibitors were all used at a concentration of 20 μg/ml except antipain, which was used at 40 μg/ml. Phenylmethyl sulfonylfluoride (PMSF; Sigma) was dissolved in a minimum amount of methanol immediately before use and used at 360 μg/ml. These protease inhibitors were all used together as a cocktail.

Diisopropylfluorophosphate (DFP, Sigma) was used at saturation by adding 10 μl per 100 ml buffer immediately before use.

Detergent Extraction of JAR Cells

For optimal extraction of undegraded gp120, JAR cell monolayers were washed three times with Dulbecco's modified PBS (DPBS) saturated with DFP. Cells were then extracted with 5 ml/150-cm^2 flask, 10 mM Tris acetate pH 8.0, 0.5% NP-40, 0.5 mM $CaCl_2$ (TNC), also saturated with DFP, at 0°C. The cells detached after a few minutes in TNC and were then agitated by vigorous pipetting for 10 min on ice. NP-40 insoluble material was removed by centrifugation at 15,000g. In some cases, JAR cells were extracted as above except that DFP was replaced with the protease inhibitor cocktail described above. JAR cells were extracted with Triton X-114 as described by Bordier [20].

Other Extractions

In other efforts to solubilize gp120, JAR cells were extraced by several procedures in the absence of detergent. Monolayers were washed with DPBS, extracted with the appropriate buffer, and centrifuged at 15,000g. The insoluble residues were reextracted with TNC as described above. The extraction conditions were as follows: 1 M urea in 10 mM Tris acetate pH 8.0, 0.5 mM $CaCl_2$ (TC); 3 M KCl in TC; 5 mM EDTA in 10 mM Tris acetate, pH 8.0; or TC (hypotonic shock) for 10 min.

Monoclonal Antibody Production

Partially purified gp120 was prepared from JAR cells by subjecting an NP-40 extract to pH precipitation with acetic acid between pH 6.0 and 5.3, $(NH_4)_2SO_4$ precipitation at 40% saturation, wheat germ affinity chromatography (the flow-through fraction contained gp120), and elution from an Affi-gel 102 (BioRad) ion-exchange column at 0.2 M NaCl. This resulted in a 200-fold purification of gp120. The final purification step involved chromatography using an anti-SFM II antibody affinity column as described previously for gp80 [1]. This preparation was mixed 1:1 with complete Freund's adjuvant (Gibco, Wilmington, DE) and injected subcutaneously at several sites along the back of a Lewis rat. One month later, the rat was injected subcutaneously with antigen mixed 1:1 with incomplete Freund's adjuvant (Sigma) followed 1 wk later by an intravenous tail vein injection with no adjuvant. The spleen was removed 4 days later and splenic lymphocytes were fused with P3-X63Ag8 mouse myeloma cells by the method of Kennett et al [21]. Hybridomas were screened initially by ELISA using detergent extracts of human epithelial cells (JAR) or human fibroblasts (WI38) as the solid-phase antigen. Those hybridomas producing antibodies positive on epithelial cell extracts and negative on fibroblast extracts were cloned by limiting dilution and screened further by immunoprecipitation, immunofluorescence, and immunoblotting. One antibody, E9, stained cell-cell boundaries of epithelial cells and immunoblotted and immunoprecipitated gp120 and gp80 in complex extracts of whole cells or conditioned medium, respectively. E9 could also be used as an immunoaffinity reagent to purify gp80 from conditioned medium as described below. None of the antibodies generated could be used successfully as an affinity purification reagent for gp120.

Purification of E9 Monoclonal Antibody

The hybridoma producing the E9 monoclonal antibody was grown in 100 ml DMEM supplemented with 10% FCS in 75-cm^2 culture flasks until the cells were no longer viable. The antibody was purified from the culture supernate on an antirat IgG

affinity column (Cappel, Malvern, PA), eluting bound IgG with 50 mM diethylamine, pH 11.0 (DEA), in phosphate-buffered saline (PBS).

Purification of gp80 by E9 Monoclonal Antibody Affinity Chromatography

Purified E9 monoclonal antibody in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) was coupled to cyanogen-bromide-activated Sepharose 4B (Pharmacia, Piscataway, NJ) at 2 mg antibody per ml packed swollen resin according to the manufacturer's instructions. Goat antihuman transferrin IgG (Cappel) was rehydrated in coupling buffer and coupled to cyanogen-bromide-activated Sepharose 4B at 10 mg antibody protein per ml swollen packed resin according to the manufacturer's instructions. Affi-blue (Sigma) was used without further treatment. In order to purify gp80, serum-free conditioned culture medium was prepared with MCF-7 cells as described [1]. Concentrated material was dissolved in TC and chromatographed sequentially on Affi-blue, antitransferrin, and monoclonal anti-gp80 (E9) columns. The 80-kd fragment did not bind to Affi-blue or antitransferrin but did bind specifically to the E9 column. The bound 80-kd fragment was eluted with 50 mM DEA pH 11.0 in TC, dialyzed against TC, concentrated, and passed over fresh Affi-blue and Sepharose 4B columns for final removal of contaminants.

Polyclonal Antisera

Anti-SFMII was produced in a goat against material shed into serum-free medium by MCF-7 cells as described previously [15]. Anti-gp80 was produced in a rabbit as described previously by immunizing with purified denatured gp80 eluted from SDS polyacrylamide gels [1]. Anti-$gp80_{E9}$, against nondenatured gp80, was produced in a rabbit by injecting native gp80 purified by E9 monoclonal antibody affinity chromatography as described above. The rabbit was injected subcutaneously at several sites along the back with approximately 5 μg purified gp80 mixed 1:1 with complete Freund's adjuvant. Three additional injections in incomplete Freund's followed at monthly intervals. Anti-$gp80_{E9}$ was particularly useful for immunoprecipitations of gp80 and gp120 whereas the original anti-gp80, made against denatured gp80, was useful for immunoblotting. Anti-SFMII, anti-gp80, and anti-$gp80_{E9}$ all disrupt cell-cell adhesion and this disruption can, in each case, be blocked by purified gp80, concentrated SFM, or an NP-40 extract of human epithelial cells.

Amino Acid Analysis

Amino acid analysis of purified gp80 was carried out on a Beckman 6300 Amino Acid Analyzer as described [22] with constant hydrolysis at 110°C in 6 N HCl for 24 hr. Phosphoamino acid analysis was done on immunoprecipitated ^{32}P-labeled protein by hydrolyzing with HCl and separating the amino acids by two-dimensional thin-layer electrophoresis as described [23,24].

Amino Terminal Sequence Analysis

Affinity-purified gp80 was precipitated with ethanol; the protein pellet was dried and dissolved in 50 μl of 88% formic acid and applied to a glass fiber filter coated with polybrene. Automated Edman degradation of gp80 was performed on an Applied BioSystems 470A gas-phase sequencer [25]. PTH amino acids obtained from the sequence were dried in vacuo and dissolved in 40 μl acetonitrile/water (1:3), and 50% of each sample was analyzed on a Zorbax column as described [26].

Labeling With ^{32}P

JAR and MCF-7 cells were grown in phosphate-free DMEM buffered with 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and with 4500 mg/l glucose (Irvine Scientific, Santa Ana, CA) supplemented with 4 mM glutamine (Gibco Labs), 25 μg/ml transferrin, 0.2 μg/ml insulin, 1.25 ng/ml selenium (ITS, Collaborative Research), and 10% dialyzed FCS. When the cells were almost confluent, the medium was replaced with serum-free medium (10 ml/75-cm^2 flask) containing 1 mCi carrier-free [^{32}P] orthophosphate (New England Nuclear, Boston, MA). After 5 hr, JAR cells were extracted with Nonidet P-40 (NP-40) as described above; MCF-7 cells were washed with phosphate-free medium and the medium replaced with phosphate-free medium without serum or transferrin. Cells were allowed to shed proteins into the medium for 48 hr as described above for nonlabeled preparations [1].

Immunoprecipitation

Detergent extracts of JAR cells labeled with [^{32}P] orthophosphate as described above were adjusted to 0.25 M NaCl, 0.3% BSA; 100 μl of this antigen was mixed with 50 μl Sepharose 4B (Pharmacia) at 4°C for 30 min to remove nonspecifically bound material. The Sepharose was separated from the antigen by microfuging for 1 min at 4°C, and the antigen was mixed with 25 μl antibody at 4°C for 1 hr. E9 monoclonal antibody was used as an undiluted supernatant and the anti-$gp80_{E9}$ antiserum was used at a 1:25 dilution. After 1 hr, 50 μl protein A or anti-rat IgG attached to Sepharose 4B was added and mixing was continued at 4°C for another hour. The Sepharose-bound immune complexes were washed five times with 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS, and the remaining pellets were boiled in sample buffer and resolved on a 7% SDS polyacrylamide gel. The gels were enhanced (ENHANCE, New England Nuclear), dried, and autoradiographed with Kodak X-Omat AR film at −70°C.

Electrophoresis and Immunoblotting

Polyacrylamide slab gel electrophoresis in the presence of SDS was done according to the procedure of Laemmli [27] with a 7% resolving gel and a 3.5% stacking gel. Gels were silver-stained as described by Oakley [28]. Two-dimensional gel electrophoresis was done as described by O'Farrell [29] with isoelectric focusing from pH 5.6 to 4.0 in the first dimension and 7% SDS-polyacrylamide slab gel electrophoresis in the second dimension. Immunoblotting was done as described by Burnette [30]. Bound antibodies were visualized by using ^{125}I-protein-A (ICN, Irvine, CA) or peroxidase-conjugated anti-IgG (Cappel). The ^{125}I immunoblots were autoradiographed by using an enhancing screen and Kodak X-Omat AR film. Those treated with peroxidase-conjugated reagents were developed with 4-chlorol-1-naphthol (BioRad, Richmond, CA).

Peptide Maps

One-dimensional peptide maps were carried out according to Cleveland et al [31] with some modifications. NP-40 extracts of JAR cells and SFM from MCF-7 cells were digested with either 0.1 μg *Staphylococcus aureus* V8-protease (Miles Biochemicals, Elkhart, IN) for 20 min at 37°C in the presence of 10 mM EDTA or with 0.1 μg chymotrypsin (Sigma) for 10 min at 37°C in the presence of 10 mM

EDTA. The resulting peptides were resolved on a 13% SDS polyacrylamide gel, transferred to nitrocellulose paper, and immunoblotted with anti-gp80 to detect immunologically cross-reacting peptides.

Endoglycosidase Digestions

Endoglycosidase F (Endo F) was obtained from Genzyme (Boston, MA) and endoglycosidase H (Endo H) from Boehringer Mannheim (Indianapolis, IN). The sample to be digested, either an NP-40 extract of cells or concentrated serum-free medium, was adjusted to 0.5% SDS and 100 mM β-mercaptoethanol and boiled for 3 min. Endo F samples were adjusted to 0.2 M sodium phosphate pH 8.6, 0.7% NP-40, 3 mM EDTA, and 0.02 U enzyme/μl. Endo H samples were adjusted to 30 mM sodium acetate pH 5.5, 0.7% NP-40, 3 mM EDTA, and 0.4 mU enzyme/μl. Samples were digested at 37°C for 12 hr and resolved on a 6% polyacrylamide SDS gel at 30 mA. Electrophoresis was continued for 1 hr after the tracking dye reached the bottom of the gel in order to maximize resolution in the molecular weight regions of interest.

RESULTS

Purification of gp80

In order to carry out the experiments reported here, especially those designed to demonstrate the direct participation of cell-CAM 120/80 in cell adhesion, it was necessary to obtain highly purified undenatured gp80. Our previously reported polyclonal anti-gp80 made against gel denatured gp80 was not useful as a purification reagent. Monoclonal antibody E9, an IgG1 that is specific for the human form of gp80, was generated as described in Materials and Methods and used to affinity purify gp80 from serum-free medium conditioned by MCF-7 cells. This antibody immunoprecipitates and immunoblots both gp80 and gp120 (Fig. 1c–e), and like polyclonal anti-gp80, stains cell-cell borders of epithelial cells (Fig. 1a,b) but not other cell types. Figure 2 demonstrates the purity of the gp80 produced by using E9 monoclonal antibody affinity chromatography. Purified gp80 blocks all of the adhesion-disrupting activity of both anti-gp80 and the original heterospecific anti-SFMII [15].

Purified gp80 Disrupts Cell-Cell Adhesion

If cell-CAM 120/80 is involved directly in cell-cell adhesion, the presence in the medium of an excess of the soluble 80-kd fragment of cell-CAM should interfere with cell-cell interactions. Figure 3 shows that this is indeed the case. Figure 3A shows the appearance of the monolayer in the absence of either the 80-kd fragment of cell-CAM or antibody to cell-CAM 120/80. Cells are tightly opposed to one another and have a typical epitheloid morphology. Figure 3B shows cells incubated with a 1:30 dilution of anti-SFMII serum, which has been shown previously to disrupt cell-cell adhesion in this system [1,15]. Cells on the monolayer have separated from one another. This antibody dilution is the minimum effective dose. Figure 3C shows cells incubated with 0.01 nM purified fragment of cell-CAM 120/80. The cells have become dissociated from one another and look much like the cells treated with antibody alone (Fig. 3B). This effect of purified gp80 is dose-dependent: 0.0025 nM has no effect, 0.005 nM causes partial dissociation, while 0.02 nM, twice the amount shown in Figure 3C, has no incremental effect. Figure 3D shows that the effect of

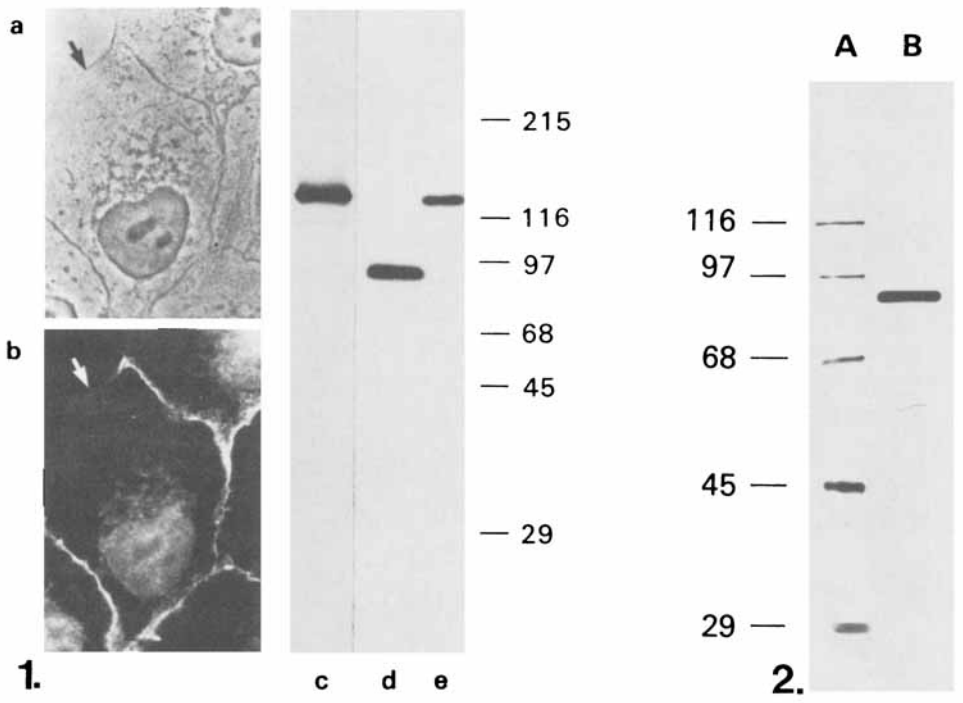

Fig. 1. Specificity of E9 monoclonal antibody. **a** and **b)** Phase and fluorescence micrographs, respectively of human epithelial cells using monoclonal antibody E9 and fluorescein-labeled antirat IgG. Cell-cell borders but not free cell borders (see arrows) are stained. **c)** Immunoprecipitation of [^{32}P] orthophosphate-labeled gp120 from NP40 extracts of JAR cells using monoclonal antibody E9. **d)** gp80 immunoblotted with monoclonal antibody E9. **e)** gp120 immunoblotted with monoclonal antibody E9. Molecular weight markers are indicated.

Fig. 2. Electrophoretic profile of E9 affinity-purified gp80. Conditioned SFM from MCF-7 cultures was cycled sequentially over Affi-blue and antitransferrin Sepharose 4B columns as described in Materials and Methods. Unbound material was cycled over 2.5 ml cyanogen-bromide-activated Sepharose 4B column to which 5 mg of E9 IgG was conjugated. The column was washed with TC and eluted with 0.05 M DEA, pH 11.5; 0.1 μg of the E9 column eluate was boiled for 3 min in sample buffer (2% SDS, 0.1 M Tris-HCl, pH 6.8, 20% glycerol, 5 mM β-mercaptoethanol) and resolved on a 7% polyacrylamide gel. The gel was silver-stained as described in Materials and Methods. **Lane A,** molecular weight markers: 116 kd, β-galactosidase; 97 kd, phosphorylase b; 68 kd, bovine serum albumin; 45 kd, ovalbumin; 29 kd, carbonic anhydrase; **Lane B,** gp80.

purified gp80 is neutralized by the combination of 0.01 nM gp80 and anti-SFMII at the same dilution (1:30) used in Figure 3B. In this panel, the cells appear as they do in the control well (Fig. 3A). The disruptive effect of purified gp80 is reversible; the cells resumed their normal morphology within 48 hr after removal of gp80.

Amino Terminal Sequence Analysis of gp80

The purified gp80 depicted in Figure 2 was used for amino acid analysis and N-terminal sequencing. The amino acid composition of gp80 (Table I) is characterized by very little methionine, cysteine, or tyrosine. There are relatively high levels of hydrophilic amino acids. Sequencing of the N-terminal 25 amino acids of gp80 (Table II) shows it to be rich in proline, indicating a lack of rigid structure in this portion of

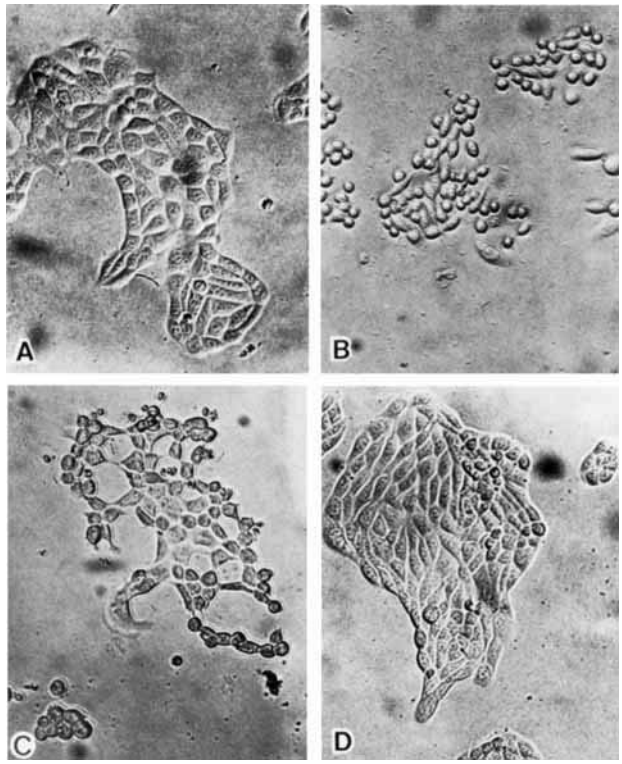

Fig. 3. Effect of purified gp80 on epithelial cell monolayers. Purified gp80, anti-gp80, or both were applied to monolayers of epithelial cells. **A)** Buffer alone; **B)** anti-SFMII; **C)** gp80; **D)** gp80 plus anti-SFMII. Purified gp80 (C) disrupts cell-cell adhesion similar to anti-SFMII (B), suggesting that it is binding to the surface of the cells. The effect of gp80 is not due to toxicity as the same amount of anti-gp80 in the presence of anti-SFMII (D) has no morphological effect on the cell. ×100.

the molecule. It was impossible to determine the identity of the N-terminal amino acid and those in positions 2 and 9 are questionable. The remaining sequence was repeated four times with no variation. The National Biomedical Research Foundation protein sequence data base, release 10, was searched and no apparent matches were found, indicating that this sequence is not homologous to any reported sequence.

Biochemical Characterization of gp120 and gp80

In the biochemical characterization of cell-CAM 120/80, we have taken advantage of the ability of polyclonal anti-$gp80_{E9}$ to immunoprecipitate both gp120 and gp80 and the ability of polyclonal anti-gp80 to immunoblot both forms. Two-dimensional gel analysis was carried out on a crude NP-40 extract of JAR cells which contains gp120 or unfractionated conditioned medium from MCF-7 cells which contains gp80. The separated samples were transferred to nitrocellulose and immunoblotted with rabbit polyclonal anti-gp80 to detect gp120 and its 80-kd fragment. Figure 4 shows two-dimensional gel analysis of gp120 (A) and gp80 (B), with isoelectric focusing in the first dimension from 5.6 to 4.0 and SDS-polyacrylamide

TABLE I. Amino Acid Composition of gp80*

Cys acid	0.7
Asp	12.5
Thr	8.3
Ser	7.2
Glu	14.7
Pro	6.2
Gly	9.8
Ala	8.1
Val	6.5
Met	0.7
Ile	4.6
Leu	6.5
Tyr	0.9
Phe	3.1
His	1.1
Lys	5.9
Arg	3.2

*The analyses were carried out as described in Materials and Methods. The results are expressed as residues per 100 amino acids and represent the means of triplicate determinations.

gel electrophoresis in the second dimension. Both species are relatively acidic with pIs of 4.7. There is only one spot for both gp80 and gp120, indicating the lack of significant charge heterogeneity typical of many glycoproteins. The 120-kd species is very sensitive to breakdown by cellular proteases and two-dimensional gel analysis also revealed one breakdown product. This protein, which is about 100 kd, is present in all preparations in which DFP is not present but disappears when we use DFP; thus it appears to be a breakdown product of gp120.

Evidence that gp80 is a cleavage fragment of gp120 derives from the experiments demonstrating that polyclonal and monoclonal antibodies prepared against the 80-kd fragment immunoblot and immunoprecipitate both gp120 and gp80. One-dimensional peptide mapping was done to further investigate the relationship of these two proteins. Detection of the peptides generated by two different proteases was accomplished by transfer to nitrocellulose and immunoblotting with polyclonal anti-gp80. This method of detection of peptides was necessary because although gp80 can be purified by E9 monoclonal antibody affinity chromatography, gp120 cannot. Therefore, comparison of peptides generated from purified proteins by silver staining was not possible; in addition, gp120 can be labeled in vivo with ^{35}S methonine or $^{32}PO_4$ but gp80 has little or no methionine and is not phosphorylated (see below). For this reason, we utilized anti-gp80 to detect immunologically related peptides. An NP-40 extract of JAR cells, which contains gp120 but no gp80, and the unfractionated conditioned SFM from MCF-7 cells, which contains gp80 but no gp120, were digested and the peptides resolved on a 13% polyacrylamide gel before transfer to nitrocellulose paper. Immunoblotting was done by using polyclonal anti-gp80 and ^{125}I-protein A. Figure 5 shows the peptide maps of gp120 and gp80 depicting immunologically cross-reacting peptides. At least five shared peptides are evident in the V8 digest of gp120 and gp80 (lanes c and d, respectively) and at least six are evident in the chymotrypsin digest (lanes e and f, respectively), supporting the hypothesis that gp80 is a breakdown product of gp120.

TABLE II. Amino-Terminal Sequence Analysis of gp80*

Cycle	Amino acid
1	—
2	Gln[a]
3	Val
4	Ile
5	Pro
6	Pro
7	Ile
8	Ser
9	Val[a]
10	Pro
11	Glu
12	Asn
13	Glu
14	Lys
15	Gly
16	Pro
17	Phe
18	Pro
19	Lys
20	Asn
21	Leu
22	Val
23	Gln
24	Ile
25	Lys

*Approximately 400 pmol of gp80 was subjected to sequence analysis in the applied biosystems model 470A sequencer as described in Materials and Methods.
[a]Not conclusive.

Two endoglycosidases were used to examine the N-linked glycosylation of human cell-CAM 120/80: Endo H, which removes only high-mannose oligosaccharides and Endo F, which removes both high-mannose and the more mature complex N-linked oligosaccharides [32–34]. Both of these enzymes require an extended incubation at 37°C, and gp120 is very sensitive to endogenous proteases, so DFP, a nonreversible serine protease inhibitor, was used to block all proteolysis of gp120. Endo H caused a slight shift in mobility (data not shown), indicating that there is a small amount of N-linked oligosaccharides of the high-mannose type in both gp120 and gp80, while Endo F treatment produced a larger shift, suggesting that the majority of the N-linked oligosaccharide in both species are of the complex type (data not shown). The extent of the molecular weight shifts following endoglycosidase treatment of the 120-kd and 80-kd forms of cell-CAM 120/80 were similar, suggesting that the majority of the N-linked oligosaccharide present is associated with the 80-kd soluble fragment of cell-CAM 120/80. These data agree with those reported for L-CAM [7].

To determine whether the 120-kd protein or the 80-kd fragment is phosphorylated, cells were incubated with [^{32}P] orthophosphate and either extracted with NP-40 to obtain gp120 or placed into serum-free medium to produce gp80. Cell-CAM 120/80 was immunoprecipitated as the 120-kd protein from the NP-40 extract or as

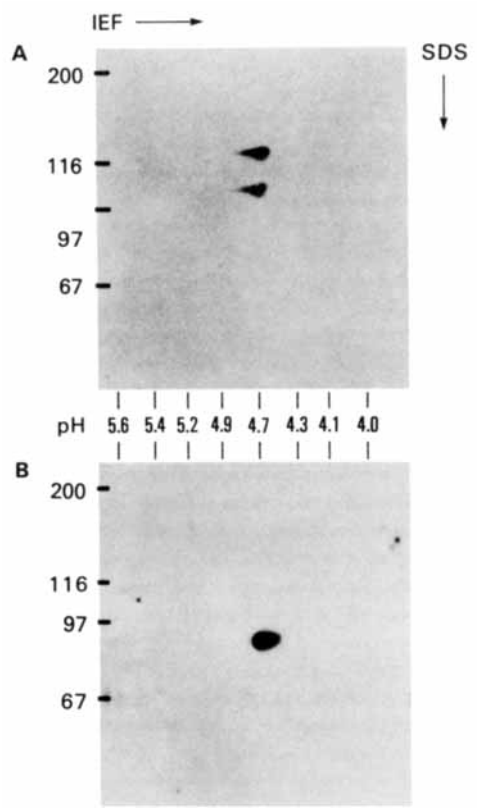

Fig. 4. Two-dimensional polyacrylamide gel analysis of gp120 and gp80. Two-dimensional gel electrophoresis was done as described in Materials and Methods with isoelectric focusing in the first dimension and SDS polyacrylamide gel electrophoresis in the second dimension. An NP-40 extract of JAR cells was used as the source of gp120. SFM conditioned by MCF-7 cells was used as the source of gp80. After running the second dimension, the gels were transferred to nitrocellulose and immunoblotted with anti-gp80 and ^{125}I protein A. **A)** Gel indicating the isoelectric point and molecular weight of gp120 and a breakdown product at about 100 kd. **B)** Gel indicating the isoelectric point and molecular weight of gp80. The pI of both species is about 4.7. pH and molecular weight markers are indicated.

the 80-kd fragment from the serum-free medium. Immunoprecipitation was done by using polyclonal antibodies prepared against the nondenatured form of gp80 (anti-$gp80_{E9}$) as described in Materials and Methods. Immunoprecipitates were analyzed by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The same strips were autoradiographed to detect ^{32}P-labeled proteins and immunoblotted with anti-gp80 to detect gp120 or gp80. Figure 6A lane a shows the immunoprecipitated NP-40 extract after SDS-polyacrylamide gel electrophoresis, transfer to nitrocellulose paper, and autoradiography for 1 hr at −70°C; gp120 is phosphorylated. Figure 6A lanes b–d show a similar immunoprecipitation of SFM. Lane b shows the total profile of phosphoproteins in the SFM after autoradiography at −70°C for 1 hr. There is a large number of labeled proteins. Lane c shows the immunoprecipitated SFM after autoradiography for 48 hr (as opposed to the 1-hr exposure that revealed gp120 in lane a); there are no phosphoproteins present. Lane d shows a Western blot of Lane c with anti-gp80 and peroxidase-labeled antirabbit IgG. Gp80 is clearly present in lane d, indicating that the lack of label in lane c is not

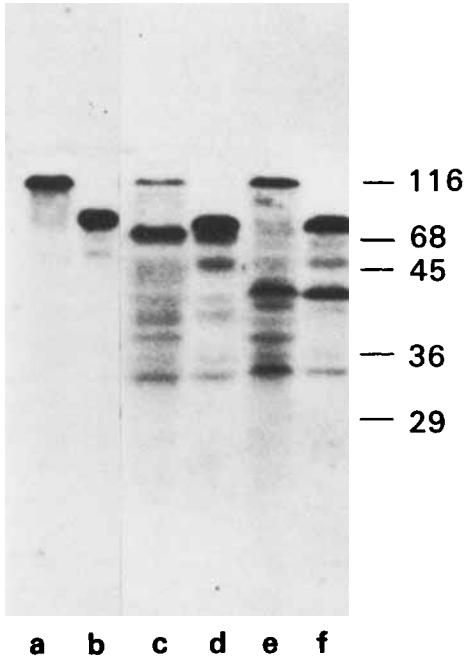

Fig. 5. One-dimensional peptide maps comparing gp120 and gp80. NP-40 extracts of JAR cells and concentrated SFM from MCF-7 cells were digested at 37°C with either 0.1 μg V8 protease for 20 min or 0.1 μg chymotrypsin for 10 min. The resulting peptides were resolved on a 13% SDS polyacrylamide gel, transferred to nitrocellulose paper, and immunoblotted with anti-gp80 to detect immunologically cross-reactive peptides. **Lanes a** and **b** are undigested controls of gp120 and gp80, respectively. **Lanes c** and **d** are gp120 and gp80, respectively, digested with V8 protease. Several peptides are shared by these two proteins. **Lanes e** and **f** are gp120 and gp80, respectively, digested with chymotrypsin. Again, several shared peptides are revealed. Molecular weight markers are indicated.

due to the absence of gp80 in the immunoprecipitated SFM. Lane d also shows a broad band at 50-kd which is the rabbit IgG heavy chain used from the immunoprecipitation. Western blot analysis of lane a (not shown) indicated that gp120 and gp80 were present in approximately equal amounts in these immunoprecipitations. Phosphoamino acid analysis of the immunoprecipitated 120-kd protein (Fig. 6B) indicated that the major phosphoamino acid is phosphoserine with no apparent phosphothreonine or phosphotyrosine.

Association of Cell-CAM 120/80 With the Cell Surface

The 120-kd form of cell-CAM 120/80 cannot be extracted from cells without the use of detergents, suggesting that it is an integral membrane protein. Extractions were attempted with 1.0 M urea, 5 mM EDTA, 3.0 M KCl, and hypotonic shock. NP-40 extractions of the pellets remaining after these extractions showed that the 120-kd protein still remained in the pellet (data not shown).

Triton X-114 has been used by some authors [20,35] to separate integral membrane proteins from hydrophilic proteins. This detergent is homogeneous at 0°C but separates into a detergent-poor and a detergent-rich phase at 20°C. Figure 7A–C is an immunoblot of an SDS-polyacrylamide gel of JAR cells extracted at 0°C with Triton X-114 and then allowed to separate into two phases. The 120-kd protein is

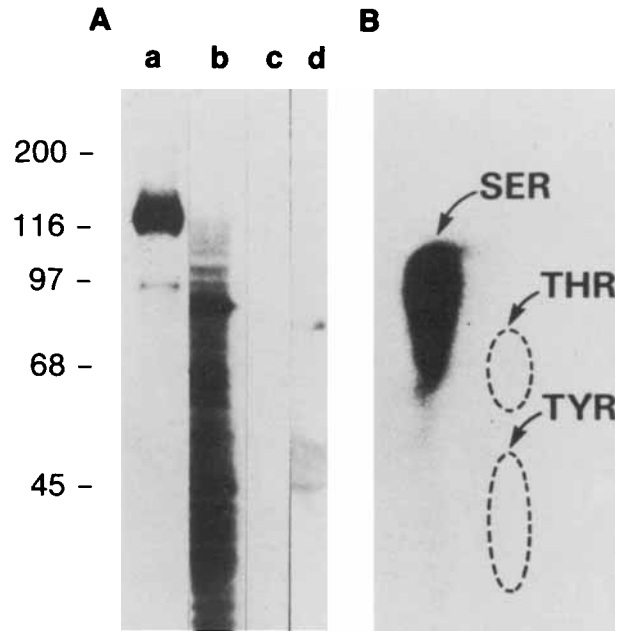

Fig. 6. Phosphorylation of gp120 and gp80. JAR and MCF-7 cells were grown in the presence of [^{32}P] orthophosphate as described in Materials and Methods. JAR cells were extracted with NP-40, and MCF-7 cells were put into SFM and allowed to shed proteins, as described in Materials and Methods. **Panel A)** The extract and the SFM were immunoprecipitated with anti-gp80$_{E9}$ and analyzed by SDS-polyacrylamide gel electrophoresis on a 7% polyacrylamide gel, transferred to nitrocellulose paper, and autoradiographed to determine the position of ^{32}P-labeled proteins. The same nitrocellulose paper was then immunoblotted with anti-gp80 and peroxidase-labeled goat antirabbit to verify the position of gp80 since it was not labeled with $^{32}PO_4$. **Lane a)** Autoradiogram of ^{32}P-NP-40 extract immunoprecipitated with anti-gp80$_{E9}$. A heavily labeled component at 120 kd is present. **Lane b)** Autoradiogram of ^{32}P-labeled SFM before immunoprecipitation. Many phosphorylated proteins are present. **Lane c)** Autoradiogram of immunoprecipitate of SFM using anti-gp80$_{E9}$. No labeled components are precipitated. **Lane d)** Immunoblot of lane c using anti-gp80 and peroxidase-conjugated goat antirabbit. Gp80 is present in the SFM and is immunoprecipitated by anti-gp80$_{E9}$ but is not labeled with ^{32}P. Molecular weight markers are indicated. **Panel B)** Phosphoamino acid analysis was done on an immunoprecipitate of ^{32}P-labeled gp120. Most of the label is found as phosphoserine.

distributed about equally into the detergent-rich (lane B) and detergent-poor (lane C) phases. In a similar extraction of SFM from MCF-7 cells, the 80-kd fragment partitioned completely into the detergent-poor phase (Fig. 7, lanes D–F).

DISCUSSION

Cell-CAM 120/80 isolated from human tumor cells is a member of an important developmentally regulated class of calcium-dependent CAMs [1]. Similar glycoproteins have been isolated from mouse teratocarcinoma cells [2–4] and chick embryonic liver [5–7]. While present on most or all cells of the very early embryo, these CAMs are restricted to epithelia in the adult organism [1,8,11].

The mechanism by which these calcium-dependent CAMs act is still not clearly understood. It has been assumed for several years, that cell-CAM 120/80, uvomoru-

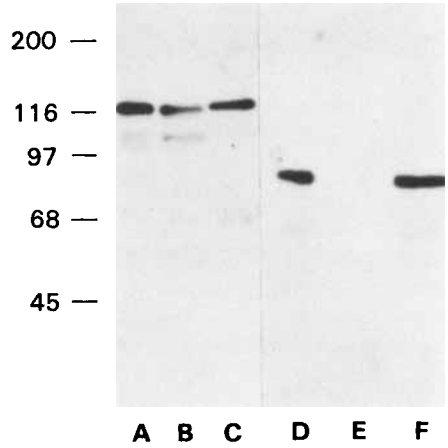

Fig. 7. Partitioning of gp120 and gp80 in Triton X-114. JAR cell monolayers were extracted with Triton X-114 at 0°C for 10 min. The temperature was raised to 20°C and the samples were microfuged for 5 min. The aqueous phase was removed and the detergent phase was washed with 10 mM Tris, 0.5 mM $CaCl_2$ at 0°C and microfuged again at 20°C. The aqueous and detergent phases were analyzed by SDS-polyacrylamide electrophoresis, transferred to nitrocellulose paper, and immunoblotted with anti-gp80. **Lane A)** Control—NP-40 extract of JAR cells. **Lanes B** and **C)** Detergent and aqueous phases, respectively, of a JAR cell Triton X-114 extract; gp120 is found in both phases. MCF-7 SFM was made 1% in Triton X-114 at 0°C and processed as described for JAR cell extracts. **Lane D)** Control—SFM before Triton X-114 extraction. **Lanes E** and **F)** Detergent and aqueous phases, respectively, on an SFM Triton X-114 extraction; gp80 is found only in the aqueous phase. Molecular weight markers are indicated.

lin, E-cadherin, and L-CAM mediate cell-cell interactions directly. However, no effect of the purified protein on cell-cell interactions has been reported. Our data show clearly that the purified gp80 fragment of human cell-CAM 120/80 is by itself capable of disrupting cell-cell interactions of MMTE cells (Fig. 3C) and that this effect can be blocked by including anti-gp80 in the medium (Fig. 3D). This is not a toxic effect since a buffer control that did not contain gp80 but had been through the same analysis and concentration procedure had no effect (Fig. 3A). Furthermore, the mixture of anti-gp80 and gp80 did not alter cell morphology (Fig. 3D). This is the first demonstration that calcium-dependent CAMs participate directly in cell-cell adhesion. The role of calcium and the nature of the molecule with which cell-CAM 120/80 interacts remain to be determined.

Other classes of calcium-dependent CAMs (eg, N-cadherin, P-cadherin) are being identified that have biochemical properties similar to epithelial CAMs but become developmentally restricted to different tissues in the adult than the epithelial calcium-dependent CAMs [38–40]. The extent of structural diversity (due to developmental age, species, tissue, and metastatic state of the source) among members of a single class of calcium-dependent CAMs and between different classes of calcium-dependent CAMs is not known. Thus, comparative information about the various CAMs should be useful in understanding their function and regulation. In the present study, using the monoclonal antibody E9, the trypsin-stable 80-kd fragment of cell-CAM 120/80 has been purified in sufficient amounts from conditioned SFM to carry out some biological and biochemical characterization studies. As expected, our biochemical characterization studies of gp80 and gp120 suggest that human cell-CAM

120/80 is very similar to L-CAM and uvomorulin. Some interesting differences exist, however, among these proteins, which are presumed to carry out homologous functions in different species.

Amino acid analysis and isoelectric focusing both suggest that the 80-kd fragment of cell-CAM 120/80 is fairly acidic and hydrophilic; gp80 has very low amounts of cysteine, methionine, and tyrosine. Sequence analysis of the N-terminal 25 amino acids of the presumed external 80-kd domain shows this portion of the protein to be proline-rich and hydrophilic. Comparable data on amino acid analysis and sequence for the mouse and chicken proteins are not currently available. Reports concerning the cloning of both L-CAM [41] and uvomorulin [42] have been published but no sequence analysis is available yet for comparison.

The relationship of this class of CAMs to the cell surface is in some dispute. The data presented here suggest that human cell-CAM 120/80 is an integral membrane protein. It continues to partition to some extent into the detergent phase of Triton X-114 even after two cycles of reextraction. This is in contrast to gp80, which enters the aqueous phase completely. These data agree with those for L-CAM but differ from those for uvomorulin, which is reported to partition exclusively into the aqueous phase [37]. Resolution as to whether the 120-kd form is or is not intercalated into the membrane awaits complete sequencing of the 120-kd polypeptide.

Cell-CAM 120/80 is both N-glycosylated and phosphorylated. The data on N-glycosylation are similar to those reported for L-CAM [7]. However, cell-CAM 120/80 is phosphorylated almost exclusively at serine while L-CAM is phosphorylated at both serine and threonine [7].

In summary, we have purified and partially characterized the biochemical and the biological activity of the 80-kd fragment of human cell-CAM 120/80. We have demonstrated that gp80 can interfere directly with cell-cell adhesion and have sequenced the N-terminal portion of the purified peptide. Biochemical characterization of cell-CAM 120/80 shows that it is very similar to L-CAM and uvomorulin. Differences do exist, however, which may be due to differences in species, tissue source, developmental age, or malignant state of the cells used as sources of uvomorulin, L-CAM, and cell-CAM 120/80.

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