A-CAM: A 135-kD Receptor of Intercellular Adherens Junctions. I. Immunoelectron Microscopic Localization and Biochemical Studies

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Abstract. The recently described adherens junction-specific 135-kD protein (Volk, T., and B. Geiger, 1984, *EMBO (Eur. Mol. Biol. Organ.) J.*, 3:2249–2260) was localized along cardiac muscle intercalated discs by immunogold labeling of ultrathin frozen sections. Analysis of this labeling indicated that the 135-kD protein, adherens junction-specific cell adhesion molecule (A-CAM), is tightly associated with the plasma membrane unlike vinculin labeling, which was present along the membrane-bound plaques of the fascia adherens. In cultured chick lens cells, A-CAM was associated with Ca^{2+}-dependent junctions that were cleaved upon a decrease of extracellular Ca^{2+} concentrations to ≤ 0.5 mM. In the chelator-separated junction, A-CAM became exposed to exogenously added antibodies or to proteolytic enzymes. Upon addition of trypsin to EGTA-treated cells, A-CAM was cleaved into three major cell-bound antigenic peptides with apparent molecular masses of 78, 60, and 46 kD, suggesting that the extracellular domain of A-CAM has a size ≥ 90 kD. Incubation of electrophoretic gels with ^{125}I-concanavalin A (Con A) indicated that one of the major Con A-binding proteins in chicken lens membranes is a ~135-kD glycoprotein that was partially purified on Con A–Sepharose column and identified as A-CAM by immunoblotting. Detergent partitioning assay using Triton X-114 biphasic system was carried out to determine whether A-CAM displays properties of an integral membrane protein. This assay indicated that the intact A-CAM molecule was recovered in the buffer phase but its cell-associated tryptic peptides, which presumably lost a great part of the A-CAM extracellular extension, readily partitioned into the detergent phase. The results obtained in this and in the following paper (Volk, T., and B. Geiger, 1986, *J. Cell Biol.*, 103:1451–1464) strongly suggest that A-CAM is a Ca^{2+}-dependent adherens junction-specific membrane glycoprotein that is involved in intercellular adhesion in these sites.

CELLULAR interactions are believed to play cardinal roles in the regulation of cell growth and development as well as in the assembly of cells into multicellular tissues and organisms (for review see references 17, 33, 54, and 55). Attempts to characterize cell contacts have revealed considerable cellular and molecular diversity of these structures. Thus, cell junctions may vary in their overall topology, fine ultrastructure, cell-type restricted occurrence, dynamics, and composition (15, 25, 31, 47). Studies on cell adhesion, carried out over the last several years, have used three major experimental approaches: (*a*) Microscopic observations (both at the light- and electron-microscope levels) have provided primary insight into the gross structural characteristics of cell adhesion to various matrices as well as to each other. (*b*) Ultrastructural studies have revealed the fine organization of cell junctions, their spatial relationship to external surfaces, and interactions with the cytoskeleton (3, 34, 37, 46). (*c*) Biochemical and immunochemical attempts have been made to identify proteins that function as "cell contact receptors" and participate directly or indirectly in the formation of defined cell adhesions. These studies have revealed several Ca^{2+}-dependent and independent cell adhesion molecules as well as substrate adhesion molecules in a large variety of cultured cells and tissues (for review see references 4, 11, 13, 14, 18, 36, 43, 45, 49, and 59). Only recently, information derived from the three experimental approaches was combined, leading to the identification of specific molecular constituents of structurally defined cell junctions such as desmosomes (8, 9, 30, 42), gap junctions (12, 28, 29, 35), and adherens junctions.

The latter type of cell contact is widely occurring in metazoan cells. It is characterized by the association of its membrane with actin-containing microfilaments through a vinculin-rich plaque structure (19, 22–26). Recent studies have indicated that at least two subfamilies of molecularly distinct adherens junctions exist. The former includes cell–cell junctions such as the zonula adherens of polarized epithelia, fascia adherens of cardiac muscle, and intercellular contacts between cultured cells of various origins (myoid cells, fibroblasts, etc.). The other group of adherens junctions consists of cell–substrate (or cell–matrix) adhesions such as focal contacts, specialized attachments to basement membranes, dense plaques of smooth muscle, etc. (3, 23, 27). Biochemical and immunocytochemical studies have

shown that these two subfamilies are molecularly distinct and, while the latter contains in its plaque both vinculin and talin, the intercellular adherens junctions are apparently devoid of talin (27). Other proteins such as the recently described 135-kD protein or uvomorulin have exhibited specific associations with the intercellular adherens junctions of lens, cardiac, and cultured primary kidney epithelial cells (135-kD protein [57]) or intestinal epithelium (uvomorulin [5]).

Besides demonstrating the specific association of the 135-kD protein with intercellular adherens junctions, we have previously shown that the antigenic epitope in intact cells is not readily accessible for labeling, unless the cells are either permeabilized or their junctions split open by the chelation of extracellular calcium ions (57). In some of its properties the 135-kD protein described by us is similar to N-Cadherin recently described by Takeichi and co-workers (32), and the relationship between the two is currently being investigated (see Discussion).

In this and the following paper (58) we examine various structural and functional properties of the 135-kD protein. In this study we have used quantitative immunoelectron microscopic labeling to determine the fine localization of 135-kD protein along the junctional membrane. We show that lowering the free Ca^{2+} concentration in the extracellular medium to <0.5 mM leads to exposure of the protein to both antibodies and proteases. We also show that the 135-kD protein is a concanavalin A (Con A)[1]-binding membrane glycoprotein. In the study reported in the following paper we show that monovalent antibodies reactive with the 135-kD protein can inhibit both cell–cell interaction and the formation of intercellular adherens-type junctions. Based on these observations we conclude that the 135-kD protein is involved in the intercellular interaction in adherens junctions and propose to name it A-CAM, namely adherens junction-specific cell adhesion molecule.

Materials and Methods

Immunoelectron Microscopy

For the immunolabeling with anti-A-CAM, freshly dissected chicken cardiac muscle was cut to small (~2 mm) pieces and fixed in a rotating shaker with 5% paraformaldehyde (PFA) containing 0.1% gluteraldehyde in 0.1 M cacodylate buffer containing 5 mM $CaCl_2$, pH 7.2 for 1 h. Alternative fixation used for vinculin and α-actinin labeling was the three stage fixation (52) including 8% PFA for 1 h followed by 5-min treatment with 20 mM ethylacetimidate in 8% PFA and finally, incubation for 1 h in 4% PFA plus 4% glutaraldehyde. The fixed tissues were washed and stored at 4°C in 0.1 M cacodylate buffer containing 0.5% PFA. Before sectioning, the tissue blocks were infiltrated for 1–2 h with 2.3 M sucrose, and ultrathin (800–1,000 Å) frozen sections cut at ~−80°C using a Sorvall MT2-B ultramicrotome equipped with cryoattachment LTC2 (50, 51). The sections were recovered on grids, washed with 0.1% glycine in PBS, conditioned two times for 10 min in 1% gelatin in PBS, washed three times with 0.1% glycine in PBS, and labeled for 30 min with the primary antibodies (anti-vinculin and anti-α-actinin were affinity-purified rabbit antibodies, while A-CAM antibodies were used as diluted ascites fluid). The sections were washed seven times for 1 min in 0.1% glycine in PBS and labeled for 30 min with the secondary antibody, either goat anti-rabbit or goat anti-mouse IgG attached to 5-nm gold particles (Janssen Pharmaceutica, Beerse, Belgium; diluted 1:10 in 20 mM Tris containing 155 mM NaCl pH 7.6). The sections were then washed (seven times for 1 min), postfixed in 2% glutaraldehyde for 3 min, washed in double distilled water, postfixed in 0.05% OsO_4 followed by washes (five times for 1 min), stained with 2% neutral uranyl acetate (10 min) in the dark, washed once in water, and stained again with aqueous 2% uranyl acetate (10 min). The sections were then embedded in methyl cellulose containing 0.1% polyethylene glycol and 0.1% uranyl acetate. The labeled sections were examined with a Philips EM 410 at 80 kV. The distances between gold particles and junctional midline were directly measured on pictures that were magnified using Graphics Calculator (Numonics Corp., Lansdale, PA).

Immunofluorescent Labeling of Ca^{2+}-depleted Cultures

Cultured chicken lens cells were seeded on glass coverslips and incubated until an epithelial monolayer was formed (57). The coverslips were briefly rinsed once with PBS and immersed in 10 mM Hepes buffer, pH 7.0 containing 150 mM NaCl, 4.5 mg/ml glucose, and different $CaCl_2$ and EGTA mixtures establishing defined-free Ca^{2+} concentrations (the computer program for the calculation of free Ca^{2+} concentration was from Dr. T. Abrams, Center for Neurobiology, Columbia University, College of Physicians and Surgeons, NY). After 30 s or 10 min of incubation with the various Ca^{2+} buffers, cells were fixed with 3% PFA in 0.5% Triton X-100 containing PBS. Immunofluorescent labeling for A-CAM was carried out as previously described (19–21).

Treatment of Cells with Trypsin

Lens cells were cultured in 3-cm culture dishes to ~75% confluence. Dishes with cells were washed with serum-free Dulbecco's modified Eagle's medium (DME) and subjected to either 0.5 mg/ml trypsin (*N*-tosyl-L-phenylalanine chloromethyl ketone [TPCK]-trypsin; Sigma Chemical Co., St. Louis, MO) or trypsin with 5 mM EGTA. The reaction was stopped by rinsing of the cells with DME containing 10% fetal calf serum and 0.1% diisopropyl fluorophosphate (Sigma Chemical Co.) The cells were removed from the culture dish using a rubber policeman, centrifuged, and boiled in SDS sample buffer. The different samples were subjected to 8% SDS PAGE and examined by blotting analyses with anti-A-CAM (see below).

Binding of Lens Membrane Glycoproteins to Con A–Sepharose

Membranes were solubilized with RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, pH 7.2) in the presence of 0.1% diisopropyl fluorophosphate (30 min at 0°C). Triton X-100 was then added to a final concentration of 1%. The extract was centrifuged in an Eppendorf microfuge and the soluble fraction was adsorbed on Con A–Sepharose column (Glycosylex A, BioYeda, Israel) in 10 mM Tris-HCl, 150 mM NaCl, 5 mM Ca^{2+}, 5 mM Mn^{+2}, and 10% BSA, pH 7.4. After 1 h of incubation at room temperature, the Con A–Sepharose was washed twice and boiled with SDS sample buffer. Samples were subjected to 8% PAGE and Coomassie Blue or silver staining. The same gels were further labeled with ^{125}I-Con A or analyzed by immunoblotting with anti-A-CAM antibodies (see below).

Purification of Lens Membranes

Purification of lens membranes was carried out according to Alcala et al. (1). Lenses from adult chickens were removed and homogenized in a dounce homogenizer in SEM buffer (0.1 M KCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 6 mM Na-phosphate pH 7.2). The homogenate was centrifuged at 17,000 rpm for 20 min in a Sorvall SS34 rotor, washed, and rehomogenized in 8 M urea in SEM buffer. The washed pellet consisted mostly of lens membrane vesicles as visualized by electron microscopy (not shown).

Gel Electrophoresis

PAGE was performed in Laemmli buffer system (39) on slab 8% polyacrylamide gels. Gels were usually stained with Coomassie Blue or silver according to Merril et al. (41). Con A–binding proteins were identified by ^{125}I-Con A overlay followed by autoradiography (7). Immunoblotting was performed essentially according to Towbin et al. (53). The protein bands were electroblotted onto nitrocellulose sheets in 50 mM Tris-glycine buffer containing 1 mM $MgCl_2$. The nitrocellulose sheets were then incubated with 10% low-fat milk (Halav-Amid, Tnuva, Israel) in PBS, and then incubated with antibody solution for ~3 h. The sheets were rinsed in the same

1. *Abbreviations used in this paper*: A-CAM, adherens junction-specific cell adhesion molecule; Con A, concanavalin A; PFA, paraformaldehyde; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

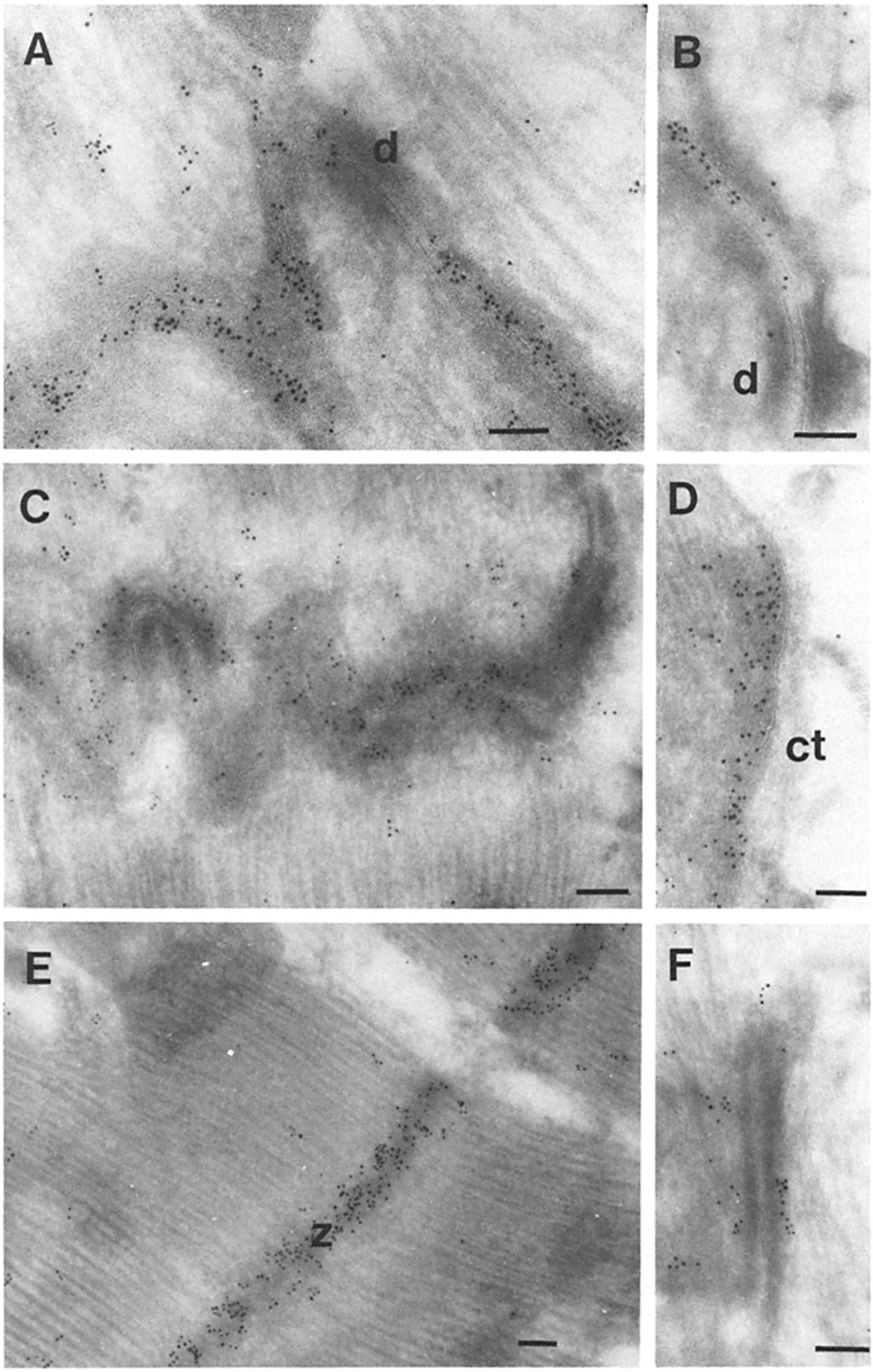

Figure 1. Immunoelectron microscopic labeling of ultrathin cryosections of chicken cardiac muscle for A-CAM (*A* and *B*), vinculin (*C* and *D*), and α-actinin (*E* and *F*). The sections shown in *A–C* and *F* include regions of the intercalated discs, while *D* shows vinculin immunolabeling along the lateral membrane of the myocytes with associated connective tissue (*ct*). Staining on Z bands (*Z*) by α-actinin antibodies is shown in *E*. Notice the absence of labeling for A-CAM in desmosomes (*d*). Bars, 0.1 μm.

buffer supplemented with 0.5% Tween 20 and incubated with ^{125}I-labeled goat anti-mouse F(ab')$_2$. After rinsing, the blot was subjected to autoradiography.

Extraction with Triton X-114 and Detergent Partitioning

The extraction with Triton X-114 was performed according to Bordier and essentially as described previously (6, 57). As will be detailed in the Results section the samples subjected to detergent partitioning included intact cultured lens cells as well as trypsin-EGTA treated cells.

Digestion of Lens Cells Extract with Neuraminidase and β-Galactosidase

Cultured lens cells were scraped off the culture dish with a rubber policeman, extracted in PBS containing 1% Triton X-100 and 0.1% diisopropyl

fluorophosphate, and boiled for 2 min. Neuraminidase and β-galactosidase (Sigma Chemical Co.) were added to the solution at final concentrations of 0.5 U/ml and 500 U/ml, respectively, and incubated for 3 h at 37°C. The samples were then boiled in SDS sample buffer and analyzed by immunoblotting with anti-A-CAM.

Immunochemical Reagents

Anti-A-CAM was a monoclonal antibody (ID-7.2.3) prepared as described previously (57). Anti-vinculin and anti-α-actinin were both affinity-purified rabbit antibodies (19, 20). Secondary antibodies used here were rhodamine-labeled goat anti-mouse $F(ab')_2$ and gold-conjugated goat anti-mouse or anti-rabbit IgG purchased from Janssen Pharmaceutica.

Results

Immunoelectron Microscopic Localization of A-CAM in the Fascia Adherens of Cardiac Muscle

To localize A-CAM in the intercalated discs at a high level of resolution we have prepared ultrathin frozen sections of chicken cardiac muscle and immunolabeled them with anti-A-CAM (ID-7.2.3 antibodies), as well as with anti-vinculin and anti-α-actinin. The results, shown in Fig. 1, demonstrate that A-CAM antibodies stain exclusively the area along the plasma membranes of the fascia adherens (Fig. 1, *A* and *B*). Desmosomes (macula adherens), which were also present in the intercalated discs, were essentially negative (see Fig. 1 *B*). Vinculin antibodies exhibited a much broader distribution of labeling largely superimposable on the junctional plaque (Fig. 2 *C*). In addition, we have frequently detected lateral, vinculin-rich plaques that were not associated with the intercalated discs but rather with collagen or other connective tissue fibers (Fig. 1 *D*). Extensive survey indicated that A-CAM was not present in those adhesions, in line with our previous results on the presence of A-CAM in cell–cell contacts exclusively (27). Labeling for α-actinin was predominantly associated with the Z-discs of the cardiac muscle (Fig. 1 *E*). Nevertheless significant labeling was also detected along the fascia adherens, where most of the label was localized along the cytoplasmic aspects of the junctional plaques (Fig. 1 *F*).

To determine quantitatively the distances between the labeling for the three proteins and the center of the junction, we have selected regions in which the fasciae adherentes were cut perpendicularly to the plane of the membranes and directly measured the distances between the center of the gold particles and the junctional midline. Nearly 600 gold particles were counted for each antigen. The results of this analysis, shown in Fig. 2, indicate that the labeling for A-CAM was confined to a narrow zone located at a distance of 50–100 Å from the center of the junction. Taking into account the fact that the intermembrane spacing in the fascia adherens is 150–200 Å (16) it appears that most of the label is aligned along the membrane proper (the position of the junctional membrane [*M* in Fig. 2] is indicated). Considering the intrinsic resolution of the method (two layers of antibodies plus gold), we suggest that the epitope on A-CAM, which recognized by the antibody is associated with the membrane itself (see Discussion). Analysis of vinculin (Fig. 2, middle) and α-actinin (Fig. 2, bottom) distributions indicated that the former had a broad peak of distribution overlapping the cytoplasmic dense plaque of the fascia adherens within 150–400 Å from the center of the junction. α-Actinin was enriched in areas outside the junctional plaque in line with the previously reported results (22).

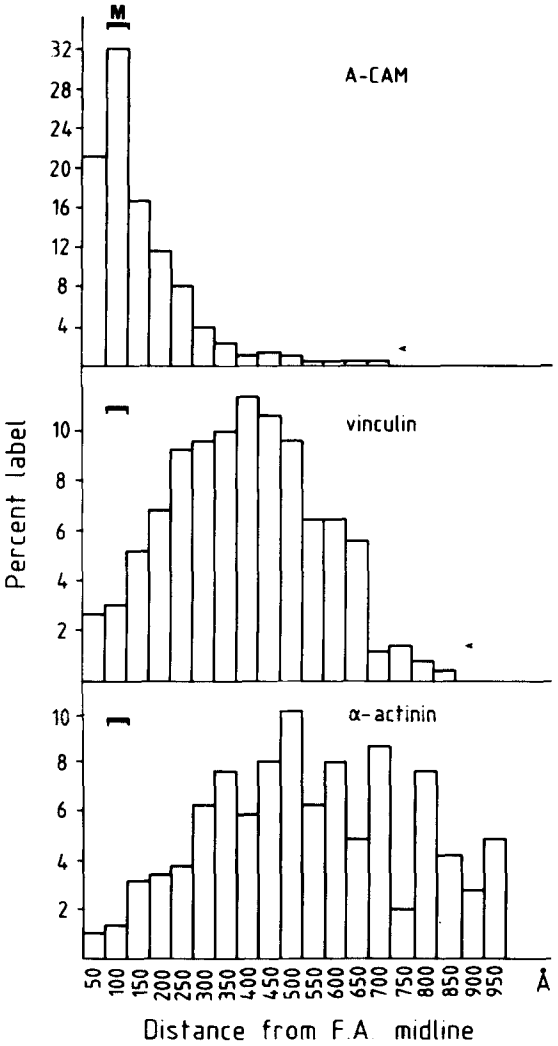

Figure 2. Histograms showing the percent of labeling for A-CAM (*top*); vinculin (*middle*); and α-actinin (*bottom*) on the cardiac frozen sections as a function of distance from fascia adherens midline. The data presented here represent values obtained from ~600 gold particles counted for each antigen. The arrowheads represent the approximate background in nonjunctional areas and the brackets (*M*) point to the location of the junctional plasma membrane.

The Effect of Extracellular Ca^{2+} Ions on the Integrity of A-CAM-containing Adherens Junctions

Intercellular adherens junctions exhibit a remarkable sensitivity to the depletion of extracellular Ca^{2+} ions. We have previously shown that a decrease in Ca^{2+} concentration to the micromolar range leads to rapid dissociation of the junction and to the exposure of A-CAM (57; see also 58). To further characterize the Ca^{2+} dependence we have incubated cultured lens cells with Ca^{2+}-EGTA buffers of various free Ca^{2+} levels for either 30 s or 10 min. The cells were then fixed-permeabilized and immunofluorescently labeled for A-CAM. The results (Fig. 3) indicated that remarkable dissociation of the junction occurred when free Ca^{2+} concen-

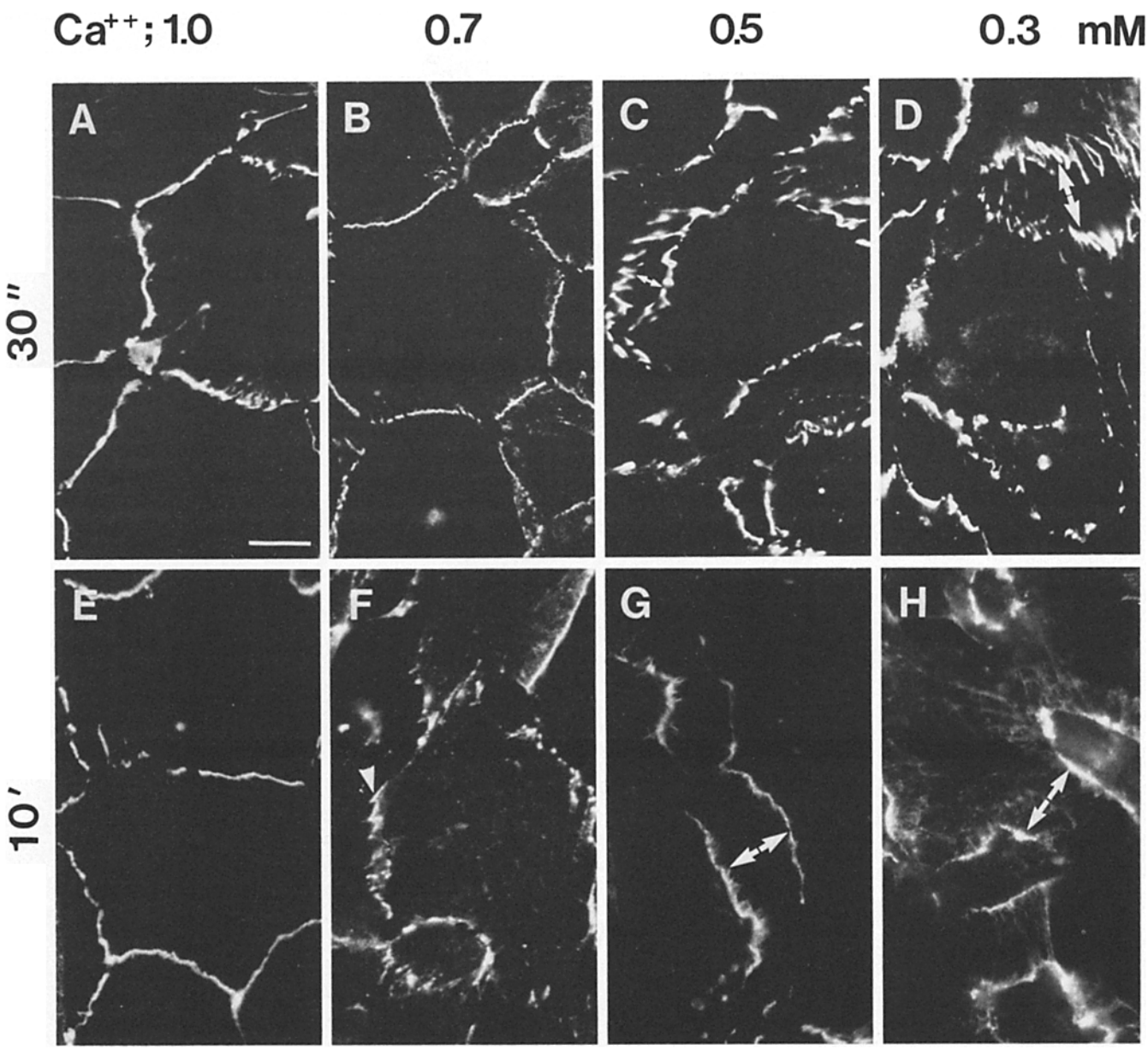

Figure 3. Immunofluorescent labeling of A-CAM on cultured lens epithelial cells treated with different Ca^{2+} concentrations after 30 s (*30"*) (*A–D*) or 10 min (*10'*) (*E–H*). The concentration of free Ca^{2+} ions are as indicated. Double-sided arrows mark the departing junctional membranes (see *C*, *D*, *G*, and *H*); arrowhead in *F* points to limited alterations in junction integrity at threshold extracellular Ca^{2+} concentration (0.7 mM). Bar, 10 μm.

trations were <0.7 mM. In fact this seems to be the minimal essential concentration since mild changes in junction integrity were noticed at this concentration after 10 min of incubation but not after 30 s (see Fig. 3 *F*). At Ca^{2+} concentrations ≤0.5 mM, rapid cleavage of essentially all junctions was noted (Fig. 3, *C*, *D*, *G*, and *H*).

We have previously shown that the cleavage of intercellular adherens junction was accompanied by exposure of A-CAM to exogenously added antibodies (57). Similarly, dissociation of these junctions rendered A-CAM sensitive to exogenously added proteolytic enzymes; cultured lens cells were treated with TPCK-trypsin in the presence or absence of EGTA. After various incubation periods at 37°C the reaction was stopped, and the cells were washed and lysed in SDS sample buffer. The presence of A-CAM or its proteolytic fragments was then monitored by immunoblotting with anti-A-CAM antibodies.

The results, presented in Fig. 4, show that A-CAM was essentially unaffected by trypsin as long as normal extracellular Ca^{2+} levels were maintained (Fig. 4, *a*, *c*, *e*, and *g*). Upon addition of EGTA and trypsin, the intact A-CAM (135-kD band) was rapidly degraded, concomitantly with the appearance of three major proteolytic fragments with apparent molecular masses of 78, 60, and 46 kD (see arrowheads in Fig. 4). Interestingly, the immunolabeling of the 78-kD fragment was far more intense than that of the intact A-CAM, probably due to steric hindrance which occurs in the intact protein and may be abolished by the trypsin treatment. The

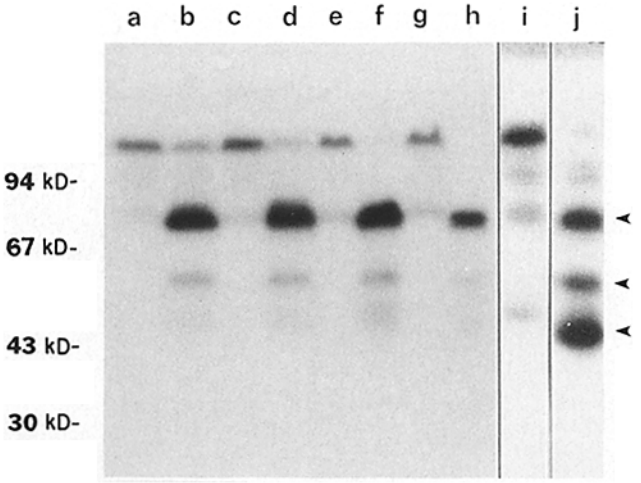

Figure 4. Immunoblot with anti-A-CAM on extract of cultured lens epithelial cells that were treated before extraction with either TPCK-trypsin (0.5 mg/ml) (lanes *a*, *c*, *e*, and *g*) or trypsin (0.5 mg/ml in the presence of EGTA [5 mM]) (lanes *b*, *d*, *f*, and *h*). Durations of incubation were 1 min (*a* and *b*); 3 min (*c* and *d*); 5 min (*e* and *f*); 10 min (*g* and *h*). Incubation of cells for 10 min with EGTA alone (lane *i*) or with 0.5 mg/ml trypsin and 5 mM EGTA for 20 min (lane *j*) are shown. Notice that A-CAM is not susceptible to trypsin unless the junction is cleaved by Ca^{2+} depletion and that A-CAM breakdown may be attributed to the exogenously added protease. The major tryptic peptides (78, 60, and 46 kD) are marked with arrowheads.

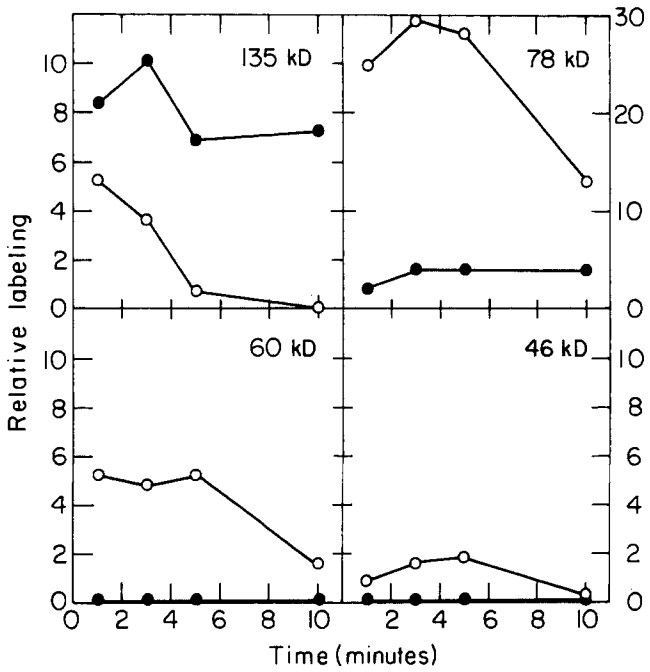

Figure 5. Densitometric quantitative analyses of A-CAM and its three proteolytic derivatives (78, 60, and 46 kD). The results were derived from densitometric scanning of the autoradiograms presented in Fig. 4. ●, labeling of each particular polypeptide after treatment with trypsin alone; ○, treatment with trypsin and EGTA. Relative labeling was measured by densitometry of the autoradiogram of the immunoblot selecting exposure times within the linear range (see Fig. 4).

Ca^{2+}-dependent and time-dependent proteolysis as well as the quantitative changes in the levels of A-CAM and its breakdown fragments are presented in Fig. 5. It is noteworthy that although we do not know, at present, the molecular relationships between the different fragments, it is reasonable to assume that the 46-kD peptide is included in the 60-kD peptide and the latter is part of the 78-kD fragment.

To determine whether the apparent sensitivity of A-CAM towards trypsin was due to changes in junction permeability or to intrinsic conformational changes in the A-CAM molecule induced in the absence of Ca^{2+} ions, we have first treated cells with EGTA until all junctions were apparently cleaved, then added excess Ca^{2+} and 0.5 mg/ml trypsin. The results indicated that once the junction was cleaved, addition of Ca^{2+} did not inhibit or modify the proteolytic fragmentation of A-CAM.

A-CAM Is a Membrane Glycoprotein

To examine some of the molecular properties of A-CAM we have isolated chick lens membranes and extracted them with RIPA buffer. The extract was then affinity-chromatographed on Con A-bound Sepharose 4B column. This chromatography brought about a considerable specific enrichment of several glycoproteins among which was a prominent band with an apparent molecular mass of ~135 kD. Identification of these glycoproteins was carried out by protein staining combined with binding of ^{125}I-Con A to the gel. Fig. 6 shows the Con A autoradiogram of the labeled gel (*a–c*) and the corresponding Coomassie Blue staining (*d–f*) where *a* and *d* show a sample of total lens membrane proteins and *b* and *e* show the Con A–bound fraction. The samples in *c* and *f* contain molecular weight markers out of which only ovalbumin polypeptide bound the ^{125}I-Con A.

Further support to the notion that the ~135 kD, Con A–binding polypeptide is related to A-CAM was obtained from immunoblotting analysis of the Sepharose-Con A-bound fraction (Fig. 7). Lens membranes were lysed in BSA containing RIPA and adsorbed on Con A column as before. In addition to the Con A–bound fraction (Fig. 7 *b*), we have examined control samples that were incubated with an irrelevant column (Sepharose-bound mouse IgG) (Fig. 7 *d*) or with Con A column in the presence of 0.02 mg/ml α-methylmannoside (Fig. 7 *c*). Examination of these samples by immunoblotting with anti-A-CAM pointed to a specific binding of A-CAM by the Con A column.

Additional support for the notion that A-CAM is a glycoprotein was obtained from neuraminidase and β-galactosidase treatment of lens cell extract, followed by immunoblotting analysis. As shown in Fig. 7 *g* the treatment with these enzymes resulted in the appearance of new antigenic band migrating as ~100-kD polypeptide (see bracket in Fig. 7) which was absent from control samples (Fig. 7 *f*).

Partitioning of A-CAM and Its Tryptic Fragments in Triton X-114 Biphasic System

Triton X-114 is a nonionic detergent with a cloud point at 20°–30°C (6). Centrifugation of detergent-containing aqueous solutions at these or higher temperatures results in phase separation into a dense detergent fraction and a lighter buffer phase.

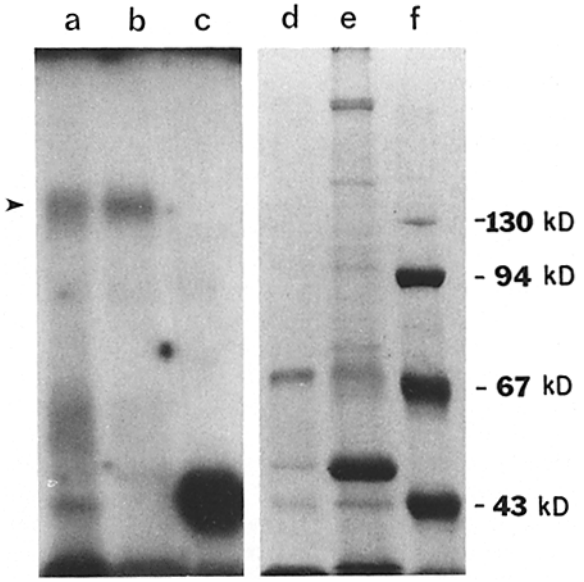

Figure 6. ^{125}I-Con A labeling (*a–c*) and Coomassie Blue staining (*d–f*) of total lens membrane extract (*a* and *d*) and the fraction retained on Con A–Sepharose column (*b* and *e*). Lanes *c* and *f* contain low molecular weight markers (from top to bottom): vinculin, phosphorylase B, BSA, and ovalbumin (only the latter is reactive with ^{125}I-Con A). Notice the abundance of a 135 kD, ^{125}I-Con A binding bands, both in the crude extract and the column-enriched fraction.

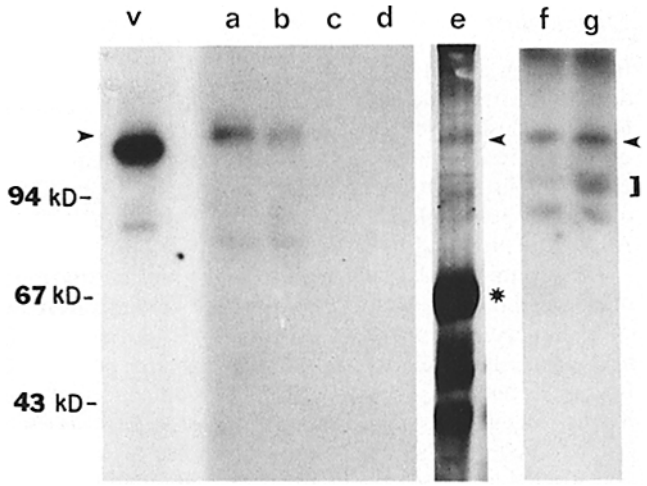

Figure 7. Immunoblotting analysis with anti-A-CAM of total detergent lens membrane extract (*a*), the fraction bound to Con A column (*b*), the fraction retained on Con A column in the presence of α-methyl mannoside (*c*), and the fraction retained on a control column of Sepharose-mouse Ig (*d*). Silver staining of the sample bound to Con A column (corresponding to lane *b*) is shown in *e*; the asterisk marks a band of BSA added to the sample to reduce nonspecific binding. A control immunoblotting with anti-vinculin is shown in lane *V*. Immunoblotting of lens cell extract treated with neuraminidase and β-galactosidase (*g*) shows the appearance of an additional band with an apparent molecular mass of ~100 kD (bracket) as compared with the control (*f*). The location of the 135-kD band is marked with arrowheads.

To examine directly the partitioning of A-CAM in this system, cultured lens cells were extracted with 1% Triton X-114 at 0°C, the extract was then clarified by centrifugation, the supernatant warmed to 37°C, and the components of the buffer (upper) phase and detergent (lower) phase analyzed by electrophoresis and immunoblotting with A-CAM antibodies. Surprisingly, the intact A-CAM was exclusively detected in the buffer phase as shown in Fig. 8 *b*. This finding could be interpreted in at least three alternative ways: (*a*) A-CAM does not contain typical membrane-spanning sequences and is possibly not embedded in the lipid bilayer; (*b*) the protein is an intrinsic membrane component but its membrane-embedded portion is not exposed in solution; (*c*) the large hydrophilic extracellular domain favors partitioning into the buffer phase fraction.

To distinguish between these possibilities we have compared the detergent-partitioning profiles of the proteolytic (tryptic) fragments of A-CAM to that of the intact protein. Cultured lens cells were treated with TPCK-trypsin in the presence of 5 mM EGTA as above after which the 135-kD band was completely degraded concomitantly with the appearance of the major tryptic peptides of 78 and 60 kD. Triton X-114 partitioning of these preparations followed by immunoblotting with anti-A-CAM indicated that unlike the intact protein, the 78-kD fragment segregated into both the buffer and detergent phases and the 60-kD peptide was mostly associated with the detergent phase. It is thus proposed that A-CAM contains a large hydrophilic domain that is largely removed by trypsin, leaving behind more hydrophobic fragments that readily partition into the detergent phase (see Discussion).

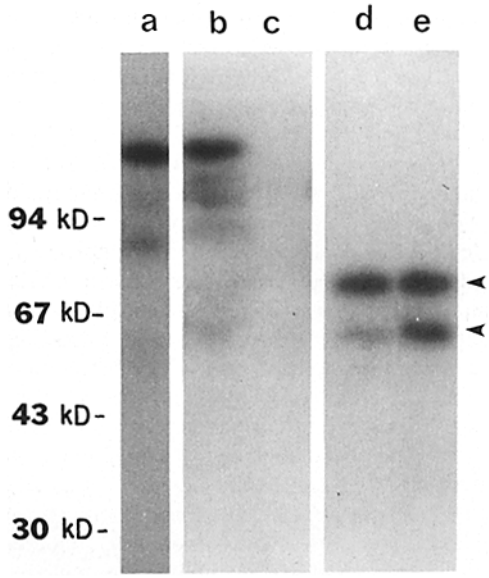

Figure 8. Immunoblotting analysis with anti-A-CAM of total Triton X-114 extract of cultured lens cells (*a*), buffer phase (*b*), and detergent phase (*c*) obtained after detergent partitioning. Lanes *d* and *e* show the partitioning profiles of the major tryptic fragments of A-CAM (78 kD and 60 kD at arrowheads) into the buffer and detergent phases, respectively. Notice that the intact A-CAM partitions into the buffer phase exclusively, while the 78-kD fragment is found both in the buffer and the detergent phase, and the 60-kD fragment is present predominantly in the detergent phase.

Discussion

In this paper and in the following one (58), we provide evidence supporting the notion that the 135-kD protein that we have previously described (57) is indeed a "contact receptor" or "cell adhesion molecule" specific for intercellular adherens junctions. Here, we would like to discuss some of the molecular properties of this protein for which the name A-CAM is proposed.

The two major issues approached in the present study are related to the fine cellular topology of A-CAM and to some of its molecular properties. The former issue was studied here using immunoelectron microscopic labeling of ultrathin frozen sections of cardiac muscle. Examination of the results, both visually (Fig. 1) or by quantitative analysis (Fig. 2), indicated that the epitope recognized by our antibodies is restricted to the plasmalemma of the fascia adherens, in line with the view that A-CAM is a component of the "membrane domain" of the junction. Examination of vinculin and α-actinin, which are components of the junctional plaque and the cytoskeletal domains respectively, provided further support of the view that the two proteins are associated with interdependent, yet spatially distinct compartments at the cytoplasmic faces of the junction (26).

It is noteworthy that the quantitative analysis performed here seems to provide reliable information at a level of resolution of ~50 Å, which is nearly four- to fivefold better than the intrinsic resolution of indirect immunogold labeling. This high level of resolution is achieved here by the statistical analysis of the label distribution profile, assuming that the antibodies have free access throughout the entire surface of the section. Moreover, to gain confidence in the significance of the results obtained by this analysis we have first examined the distribution profile of fewer particles (450–500), and found that an increase in the size of the experimental sample of up to ~600 particles did not significantly affect the results.

Along the junctional membrane, the epitope recognized by A-CAM antibody is apparently exposed at the external cell surfaces. It has been previously shown that viable or fixed (but not permeabilized) cells show little or no labeling with anti-A-CAM (57). Yet brief treatment of viable cells with Ca^{2+} chelator (EGTA) leads to a rapid exposure of the epitope. This property has been previously established by immunofluorescence microscopy and is further documented in the following paper by immunoelectron microscopy (see Fig. 3 *E* in reference 58). Here we have shown that depletion of Ca^{2+} from the extracellular medium render A-CAM sensitive to exogenously added trypsin, suggesting that this sensitivity is due to an actual dissociation of the junction. One may consider two possible mechanisms to be involved in the acquisition of such sensitivity: The first may depend on the EGTA-induced exposure of A-CAM which was hindered within the intact junction, and the second may be related to an exposure of the cleavage site(s) during the dissociation of A-CAM from its corresponding binding molecule on the neighboring cell's membrane.

Another possibility, namely that the depletion of Ca^{2+} ions induces conformational changes in A-CAM molecule which render it trypsin sensitive, seems unlikely since once the junction was cleaved A-CAM was susceptible to trypsin regardless of whether Ca^{2+} ions were present or not. Interesting features of this dissociation are the speed of cell separation and the strict Ca^{2+}-concentration dependence of the process. Usually 30 s were necessary for initial visible opening-up of the junctions after which remarkable and rapid cell contraction was noted. Moreover, in lens cells incubated with 0.7 mM free Ca^{2+} concentration the junctions were hardly affected (limited effect was noted after prolonged incubation) but at 0.5 mM free Ca^{2+} concentration essentially all junctions were rapidly cleaved. These EGTA concentration- and time-dependent dissociations are apparently not cell-type specific and manifested by other A-CAM containing cells (such as primary kidney epithelial cells, cultured from 15-d-old chick embryos) (Elson, A., T. Volk, and B. Geiger, unpublished observations) pointing to a cooperative nature of the intercellular adhesion in this junction. We would not like to further extend here the discussion on Ca^{2+}-dependence. It is suffice to mention that the phenomenon is shared by desmosomes and cell–cell adherens junctions (38, 56) and that in several experimental systems the entire plaque and cytoskeletal domains detach, as one unit from the membrane after Ca^{2+} removal (reference 56 and see also Discussion in the following paper [58]).

There are several additional interesting aspects to the EGTA-trypsin experiments. First, they provide additional evidence for the surface location of A-CAM; the principle of Ca^{2+}-dependent protection from proteolysis have also been successfully used by Takeichi and co-workers (48) for the identification of Ca^{2+}-dependent cell adhesion molecules (defined by them as "cadherins"). A second aspect is related to the fine location of the epitope along the A-CAM molecule. We have shown here that there are three major antigenically-competent cell-associated fragments of A-CAM obtained after partial tryptic treatment of cells. These fragments probably retain their membrane anchorage domain. Knowing that the smallest cell-bound fragment that we detect has a molecular weight of ~46,000, we may conclude that the extracellular domain of A-CAM is larger than 90 kD and that the antigenic epitope recognized by our antibody is located proximally to the membrane proper. It is interesting to note that the large fragment of ~78 kD is similar in its approximate molecular weight to the major tryptic fragments of uvomorulin, L-CAM, Cell-CAM 120/80, and Arc-1 (4, 10, 11, 18, 36, and see Discussion in the following paper [58]). Further characterization of A-CAM with respect to the location of the cell-binding site, the actual size of the membrane-intercalated part, and the possibility that the molecule also contains a cytoplasmic portion is now in progress.

An unexpected observation obtained in the trypsin-EGTA experiment is the much higher extent of labeling of the 78-kD polypeptide in immunoblots as compared with that obtained with the intact molecule (see Fig. 4). This augmentation could not be attributed to quantitative differences since all the 78-kD peptide present in these samples was derived from the intact 135-kD A-CAM. It was thus concluded that the removal of a ~55-kD external region increases the accessibility, and hence the binding affinity of the antibodies to the residual fragment. This property of A-CAM may also be related to the low efficiency of immunoprecipitation of the intact molecule by ID-7.2.3 antibodies. Attempts are now being made to determine whether the proteolytic fragments may be more readily immunoprecipitated.

The protease treatment has provided an additional clue as to the amphipathic nature of A-CAM. The detergent-

partitioning experiment using Triton X-114 biphasic system have indicated that the intact A-CAM, which was expected to display properties typical of an integral membrane component, have partitioned into the buffer phase. Experience in other laboratories have well established that integral membrane proteins such as bacteriorhodopsin, cytochrome *c* oxidase, human erythrocyte acetylcholine esterase, etc. (6), segregate predominantly into the detergent phase of such a system. The results of Triton X-114 partitioning should, however, be evaluated with caution. For example, Maher and Singer have recently shown that acetylcholine receptor, which is certainly an integral membrane protein with typical membrane-spanning amino acid sequences (2), is recovered in the buffer phase of the Triton X-114 system (40). This anomaly was explained by the putative existence of hydrophilic regions along the transmembrane domain of the receptor, facing the ion channel. We have no information as to the possible presence of similar hydrophilic transmembrane regions in A-CAM, but in our case it could be demonstrated that the large extracellular extension of A-CAM has a dominant effect on the results of the detergent partitioning experiment. In contrast to the intact A-CAM, the tryptic 78-kD and especially the 60-kD fragments (which are a part of the 78-kD polypeptide and presumably retain the membrane-binding region) segregated into the detergent phase. We suggest therefore that the distribution and relative size of the extracellular domains of membrane proteins should be considered as important factors in evaluating Triton X-114 partitioning results in general. Interestingly, similar experiments have indicated that another cell adhesion molecule, namely uvomorulin, also partitions into the buffer phase of Triton X-114 biphasic system. This result was interpreted by Peyrieras and co-workers (44) as indicating that uvomorulin is not an integral membrane protein. This is in contrast to reports of other groups suggesting that the closely related L-CAM in liver membrane (for discussion see reference 58) has the properties of an intrinsic membrane protein (10). In view of the results presented here we propose that detergent partitioning experiments with uvomorulin will be extended and that the properties of the cell-bound residue after proteolytic cleavage of the extracellular hydrophilic domain be carried out.

The last aspect to be discussed here is related to the glycoprotein nature of A-CAM. In the absence of pure protein, suitable for direct analysis, we have used here two experimental approaches to study this aspect: binding to Con A and treatment with neuramindase and β-galactosidase. Experiments along the former approach have indicated that lens membranes contain a major Con A–binding glycoprotein with an apparent molecular mass of 135 kD. This protein could be identified as A-CAM by immunoblotting. The binding of A-CAM to Con A was apparently specific in as much as it could be inhibited by α-methyl mannoside and did not occur when wheat germ agglutinin or mouse IgG columns were used instead of Con A column. Conceptually, the lectin binding could be used as a major step in the purification of A-CAM but our experience so far has indicated that the recovery of A-CAM from Con A columns was not satisfactory. Attempts to replace the Con A by other lectins (e.g., lentil lectin) are presently being carried out.

To corroborate the lectin studies we have examined the effect of specific glycosidases on the electrophoretic migration of A-CAM. For that purpose we have used both endoglycosidase H and a neuraminidase–β-galactosidase mixture. The former treatment required relatively long incubations that resulted in a considerable proteolysis of A-CAM (not shown) and rendered the interpretation difficult and equivocal. The neuraminidase–β-galactosidase treatment, on the other hand, effectively converted part of the 135-kD band to lower molecular weight (~100 kD) species.

In conclusion, the data presented have established the fine localization of A-CAM and provided some information on its molecular properties. A major aspect that has not been dealt with here concerns the function of A-CAM in the adherens junction. In the following paper (58) we will present evidence supporting the notion that A-CAM is directly involved in intercellular adhesion.

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