Introduction

A-CAM is a Ca\textsuperscript{2+}-dependent cell adhesion molecule which mediates cell-cell contact in adherens type junctions (AJ, Volk and Geiger, 1984; Volk and Geiger, 1986a,b). Recent immunocytochemical and molecular genetic data have indicated that A-CAM is a member of the cadherin family of cell adhesion molecules (for review see Takeichi et al., 1985; Takeichi, 1988), and is essentially identical to N-cadherin (Hatta et al., 1985, 1988; Duband et al., 1987, 1988). Immunolabeling of A-CAM in a variety of adult and embryonic tissues and in cultured cells showed it to be enriched at AJ which are also enriched in vinculin, \( \alpha \)-actinin and actin (Volk and Geiger, 1984, 1986a). Moreover, biochemical data along with the complete sequencing of N-cadherin cDNA (Hatta et al., 1988) revealed the general structural features of this molecule. Similar to other cadherins, it displays an apparent molecular weight of 127-135 kDa and its polypeptide chain contains a single membrane spanning region located between a large N-terminal extracellular domain and a smaller, C-terminal cytoplasmic tail (Takeichi, 1988). The various cadherins may be involved primarily in homophilic interactions mediated through their external domains, and may interact with the microfilament system at the cytoplasmic faces of the junctional membrane through their C-terminal regions (Hirano et al., 1987; Nagafuchi and Takeichi, 1988).

 Localization of A-CAM and other CAMs in developing embryos have indicated that their spatiotemporal expression is closely related to major morphogenetic events, including the assembly and folding of epithelia, cell migration and immobilization (Edelman, 1985; Hatta and Takeichi, 1986; Duband et al., 1988). Although A-CAM is expressed in a large variety of cell types in the embryo in all three germ layers, it is most prominent in the developing neural tube, heart, ectodermal placodes, somites and other mesoderm-derived epithelia. Moreover, epithelium-to-mesenchyme conversions, such as the development of sclerotome or of migratory neural crest cells, are accompanied by a marked decrease in A-CAM expression (Duband et al., 1988). These observations, together with a detailed information on the spatiotemporal expression of other CAMs during embryogenesis (see, for review Edelman, 1985; Takeichi, 1988) strongly suggest that the differential expression of these molecules indeed plays a cardinal role in embryonic morphogenesis. The modes by which A-CAM expression and activity are modulated during development have not yet been characterized in detail, though preliminary results of \textit{in situ} hybridization (Ffrench-Constant and B. Geiger, unpublished data) indicate that...
the levels of N-cadherin/A-CAM mRNA closely correspond to the patterns obtained by immunocytochemical labeling.

Results presented in this paper suggest that A-CAM activity may be down-regulated post-translationally, via its degradation by membrane-associated proteinases. We report here on two distinct proteolytic activities which lead to the apparent cleavage of A-CAM in cultured chicken lens cells. The first, which take place in the presence of Ca\textsuperscript{2+}, results in a constitutive release into the culture medium of a 9.7-kDa polypeptide (apparent molecular weight) corresponding to the extracellular domain of A-CAM. The other proteolytic activity leads to cleavage of A-CAM in the extracellular domain of the molecule, leaving a 78-kDa fragment associated with the cell surface. Immunocytochemical labeling of chicken embryos with a pair of monoclonal antibodies that distinguishes intact from cleaved A-CAM molecules suggests that the disappearance of A-CAM from the sclerotomal portion of dissociating somites may initially involve a proteolytic process.

MATERIALS AND METHODS

Purification of A-CAM

A-CAM was purified from chicken heart membranes and used as an immunogen for the production of a new set of monoclonal antibodies (mAbs) which are described here for the first time. The purification included the following steps. Heart membranes were isolated from chick cardiac muscle by a modification of the method of Colaco and Evans (1981, 1982) as previously described (Volk and Geiger, 1984). Heart slices were suspended in approximately 10 vol of buffer A (10 mM Tris-Histidine buffer, 20 mM Na-pyrophosphate, pH 7.4) and homogenized for 10 min in Ultra Turrax homogenizer at medium speed. EGTA was added to a final concentration of 0.1 mM and the solution was further homogenized for 5 min in a loose Dounce homogenizer. CaCl\textsubscript{2} was then added up to 0.1 mM, the extract was filtered through cheese cloth and centrifuged in a refrigerated Sorvall SS 34 rotor at 3000g for 10 min. The pellet was washed three times with buffer A, suspended in buffer B (10 mM Tris-histidine buffer, 0.6 M KCl, 8% sucrose, pH 7.4), and incubated at 4°C for 18 hr. The pellet was then collected following centrifugation as above and was extracted for 2 hr with 1% Triton X-100, 140 mM NaCl, and 50 mM Tris, pH 7.4, at 4°C. The insoluble residue was discarded and the supernatant was kept at −70°C until further processed. All the solutions and buffers contained 2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma, St. Louis, MO).

A-CAM was purified by affinity chromatography on Sepharose-bound anti A-CAM (mAb ID-7.2.3, see Volk and Geiger, 1984). These antibodies were isolated from hybridoma supernatants by immunoadsorption on goat anti-mouse F(ab')\textsubscript{2} antibodies coupled to Sepharose-polyacrylhydrazide (2 mg goat anti-mouse/g gel). Hybridoma supernatant (150 ml) was passed through 10 ml of the anti-mouse column. Anti A-CAM antibodies were eluted with 0.15 M NH\textsubscript{4}OH. These antibodies were dialyzed extensively against PBS and coupled to a Sepharose-polyacrylhydrazide column (0.9 mg/g gel). Triton X-100 extract of heart membranes was incubated for 1 hr with continuous shaking with the Sepharose-bound ID-7.2.3 mAb. The immunoadsorbent was washed twice with PBS containing 1% Triton and twice with PBS.

The bound A-CAM was then eluted with 10 M urea, dialyzed against 10 mM phosphate buffer (pH 7.2), lyophilized, dissolved in water, and injected into Balb/c mice for the production of monoclonal antibodies. Positive clones were selected mainly according to the pattern of staining of chick lens and heart tissues and immunoblotting analysis. The new hybridoma clones isolated and used in this study are designated mAbs CC-11, FA-5, GB-9, and BB-6. (These antibodies are now available through Sigma Immunochemicals, USA).

Gel Electrophoresis and Immunoblotting

Polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (1970) on slab 8% polyacrylamide gels. Gels were routinely stained with either Coomassie blue or processed for silver staining according to Merril et al. (1981). Immunoblotting was performed essentially according to Towbin et al. (1979): The protein bands were electroblotted onto nitrocellulose sheets in 50 mM Tris-glycine buffer, pH 8.6, containing 1 mM MgCl\textsubscript{2}. The nitrocellulose sheets were incubated with 10% low-fat milk in PBS, and then with the primary antibody solution at an appropriate dilution for 2 hr. The sheets were rinsed in 10% low-fat milk in PBS supplemented with 0.05% Tween-20 (Sigma, St. Louis, MO) and incubated with \textsuperscript{125}I-labeled goat anti-mouse F(ab')\textsubscript{2}. After extensive rinsing, the blots were subjected to autoradiography.

Preparation of Primary Chick Lens Cell Cultures

Lenses of 6- to 8-day-old chick embryos were dissected, suspended in trypsin/EDTA solution and passed through a 27-gauge syringe needle. The suspended lens cells were then centrifuged, rinsed with DMEM containing 10% FCS, and plated either onto 35 × 10 mm culture dishes (Falcon, USA) or seeded in 30 μl drops on glass coverslips. The lens cells formed typical epithelioid islands that were essentially free of fibroblasts (Volk and Geiger, 1984).
Treatment of Cells with Proteinases

Lens cells in 35 x 10 mm Falcon culture dishes were washed with serum-free DMEM and subjected to 0.1 mg/ml trypsin (TPCK-Trypsin; Sigma, USA) in serum-free DMEM or in DMEM containing 3 mM EGTA. The reaction was stopped by rinsing the cells with DMEM containing 10% fetal calf serum and diisopropyl fluorophosphate (Sigma, St. Louis, MO). The cells were removed from the culture dish using a rubber policeman, centrifuged, and immediately boiled in SDS sample buffer. Other proteinases that were used were V8 proteinase (Sigma, St. Louis, MO) and Bromelain (Sigma, St. Louis, MO), both at a concentration of 0.1 mg/ml.

Immunochemical Reagents

The antibodies used in this study included:

(a) Anti A-CAM monoclonal antibodies. MAb ID-7.2.3 was prepared as previously described (Volk and Geiger, 1984) and used primarily as hybridoma supernatant. Monoclonal antibodies CC-11, FA-5, GB-9, and BB-6 were prepared by injection of partially purified A-CAM into mice as described above. All these new antibodies react with the N-terminal region of the A-CAM molecule; in this study only results with mAbs cc-11 will be shown.

(b) Antibodies reactive with the C-terminal 24-amino acids of A-CAM. A synthetic polypeptide consisting of the C-terminal amino acids of N-cadherin (DYLNDWGPRFKKLDMYGGD; for the sequence see Hatta et al., 1988) was synthesized, coupled to Keyhole Limpet Hemocyanine and injected into rabbits. The antibodies (R-156) reacted with A-CAM as well as with several other cadherins, as will be described in detail elsewhere. These antibodies were used to recognize A-CAM fragments that contain the intact C-terminus.

(c) Goat anti-mouse and goat anti-rabbit antibodies. As secondary antibody reagents, we have used affinity-purified goat anti-rabbit Ig and goat anti-mouse F(ab')2. These antibodies were coupled to rhodamine-lissamine sulfonyl chloride or to dichlorotriazinyl amino fluorescein as described (Brandtzæg, 1973; Geiger and Singer, 1980; Avnur and Geiger, 1981).

Immunoelectron Microscopy

Gelatin solution (10%) was layered on coverslips and fixed for 12 hr with 0.5% glutaraldehyde in phosphate buffer. The gelatin films were incubated for 5 min with 2% NaBH₄, washed extensively with H₂O, sterilized under uv light, and conditioned with FCS. Cells cultured on the gelatin film were fixed for 30 min with 3% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M cacodylate buffer, washed, layered with a thin layer of 10% gelatin, and refixed. The embedded monolayer was then incubated with 2.3 M sucrose in PBS, frozen in liquid N₂ and sectioned in a Reichert-Jung ultra-cryotome FC-4D at -96°C. Immunolabeling was carried out as previously described (Volk and Geiger 1986a).

RESULTS

Trypsin Sensitivity of A-CAM: The Involvement of Ca²⁺ Ions

As previously described, the addition of trypsin in serum-free Ca²⁺-containing DMEM to cultured lens cells for a few minutes had no significant effect on the integrity of A-CAM (Volk and Geiger, 1986a; see also Fig. 1). When Ca²⁺ ions were depleted from the culture medium by EGTA (Low Ca²⁺, <0.3 mM (LCa²⁺)), however, trypsin caused a rapid cleavage of A-CAM, leaving on the cell surface a major 78-kDa immunoreactive fragment. As previously shown, longer incubations with
trypsin-EGTA resulted in the formation of additional fragments with apparent molecular weights of about 60 and 46 kDa (Volk and Geiger, 1986a). These residues were all reactive with antibody ID-7.2.3 (Fig. 1). The continuous depletion of Ca\(^{2+}\) ions from the medium was not essential for the trypsin-mediated cleavage of A-CAM, as pretreatment of lens cell cultures with 3-5 mM EGTA in DMEM followed by rinsing with normal DMEM (NCa\(^{2+}\)) and immediate addition of trypsin resulted in cleavage of A-CAM (Fig. 1).

The tight association of the 78-, 60-, and 46-kDa tryptic fragments of A-CAM with the cell surface initially suggested that they are produced by sequential cleavage at three distinct extracellular sites, retaining the transmembrane domain of the molecule (see Volk and Geiger, 1986a). The present results indeed confirm that the 78-kDa fragment contains the C-terminus of A-CAM since it reacted with antibody R-156, directed against the C-terminal 24 amino acids of N-cadherin (Fig. 1). Moreover, mAb CC-11 and other mAbs which react strongly with the intact molecule (see Materials and Methods) failed to bind to the 78-kDa fragment since they presumably bind to the cleaved-off N-terminal area (Fig. 2). These antibodies efficiently disrupt adherens junctions formed between cultured lens cells and are, in this respect, similar to the NCD-2 antibody described by Hatta et al. (1985). Surprisingly R-156 did not react with the 60- and 46-kDa tryptic fragments since they presumably bind to the cleaved-off N-terminal area (Fig. 2), suggesting that the 78-kDa fragment was further cleaved at its cytoplasmic domain, probably by an endogenous proteinase (see Discussion). Assignments of antigenic epitopes to different regions along A-CAM are further supported by a direct analyses of the immunoreactivity and functional properties of the protein products of truncated N-Cadherin transfected into CHO cells (Ginsberg and Geiger, unpublished data).

**Release of a 97-kDa, A-CAM Related Polypeptide, from Cultured Lens Cells**

Another A-CAM-related polypeptide was revealed by immunoblot analysis with antibody ID-7.2.3, of the culture medium of lens cells. The released polypeptide has an apparent molecular mass of ~97 kDa (migrating slightly above phosphorylase-b) and its levels in the medium increase with incubation time (Fig. 3). Furthermore, immunoblotting analysis indicated that this polypeptide reacts with all our monoclonal antibodies but not with the rabbit antibody R-156 reactive with the C-terminal 24 amino acids (Fig. 3). It seems most likely...
that the 97-kDa released molecule corresponds to the entire extracellular domain of A-CAM or a major portion of it.

To determine whether the released polypeptide was indeed derived from surface-associated A-CAM, we have carried out the following experiment: Chicken lens cells were briefly treated with trypsin-EGTA (5 min at 37°C). This short treatment resulted in the essentially complete cleavage of the intact A-CAM into the 78-kDa fragment (Fig. 4). The treated cultures were then washed with NCa²⁺ medium, supplemented with 1% FCS, and further incubated for 5 or 10 hr. Aliquots of the culture medium were then examined by immunoblotting using antibody ID-7.2.3. Pretreatment with trypsin-EGTA (but not with EGTA alone) drastically suppressed the release of the 97-kDa polypeptide into the culture medium (Fig. 4), consistent with the idea that this fragment is indeed derived from a membrane-bound, trypsin-EGTA-sensitive precursor (see Discussion).

Cleavage of A-CAM by an Endogenous Proteinase in Low Ca²⁺ Medium

As shown above, surface-associated A-CAM was readily cleaved by trypsin either in LCa²⁺ medium or when applied immediately following EGTA treatment (Fig. 1), but was not rapidly altered by depletion of Ca²⁺ ions alone. However, prolonged incubation of cultured lens cells in LCa²⁺ medium, without the addition of any exogenous proteinase, resulted in a progressive cleavage of the molecule, leaving on the cell surface a 78-kDa residue, which was essentially indistinguishable from the trypsin-cleaved fragment described above. This was manifested by the size of the fragment and its reactivity with antibody R-156 (anti C-terminus) but not with the mAbs reactive with the N-terminal region of A-CAM (Fig. 5). The 78 kDa cleavage product was already apparent after 30 min of incubation in LCa²⁺ medium and the intact A-CAM essentially disappeared following 24 hr of incubation (Fig. 5). Switch of EGTA-treated cultures into NCa²⁺ medium resulted in a gradual reappearance of the intact A-CAM concomitant with the disappearance of the 78-kDa fragment.

Attempts to inhibit the formation of the 78-kDa fragment by proteinase inhibitors were partially successful. As shown in Fig. 6, the addition of anti-proteinases (leupeptin (100 μg/ml), elastinal (100 μg/ml), bestatin (100 μg/ml), pepstatin (100 μg/ml), chymostatin (100 μg/ml), and antipain (40 μg/ml)) partially suppressed the conversion of the intact A-CAM into the 78-kDa polypeptide.

![Figure 4](image_url)

**Fig. 4.** The effect of EGTA–trypsin pretreatment on the subsequent release of the 97-kDa, A-CAM-related polypeptide. Subconfluent cultured lens cells in 35-mm dish were incubated in low Ca²⁺ medium (3 mM EGTA) (E), or in the same low Ca²⁺ medium containing 0.1 mg/ml trypsin for 5 min. The cells were then washed and further incubated in 1 ml normal medium (containing 10% FCS) for 5 and 10 hr. Aliquots of the medium were boiled in sample buffer and analyzed by immunoblotting with antibody ID-7.2.3. Control lane (C) represents nontreated cells. Cells pretreated with trypsin–EGTA for 5 min as above (TE), and recovered in normal medium for 0, 5, and 10 hr, were lysed in sample buffer and similarly analyzed by immunoblotting with ID-7.2.3. Notice that pretreatment with trypsin–EGTA strongly suppressed the release of the 97-kDa polypeptide to the medium.

![Figure 5](image_url)

**Fig. 5.** Cleavage of A-CAM in low Ca²⁺ medium. Cultured lens cells (subconfluent culture in 35-mm dishes) were incubated as indicated (0-24 hr) in 1 ml DMEM containing 5 mM EGTA, lysed in sample buffer and examined by immunoblotting using antibodies ID-7.2.3, CC-11, or R-156. In addition, cells preincubated for 24 hr in LCa²⁺ medium were switched to NCa²⁺ levels (recovery) for the indicated periods. Notice that in low Ca²⁺ the intact A-CAM gradually disappeared until it was hardly detectable (24 hr). Concomitantly a 78-kDa polypeptide appeared in these samples. The 78-kDa polypeptide was recognized by antibodies ID-7.2.3 and R-156 but not by CC-11 (similar to the major cleavage product with trypsin in the presence of EGTA).
FIG. 6. Partial inhibition of A-CAM proteolysis in low Ca\(^{2+}\) medium by a mixture of proteinase inhibitors. Cultured lens cells were incubated in low Ca\(^{2+}\) medium for 20 hr (E). One hour prior to addition of EGTA, a mixture of proteinase inhibitors was added (E + iP). This mixture contained: leupeptin (100 \(\mu\)g/ml); elastinal (100 \(\mu\)g/ml); bestatin (100 \(\mu\)g/ml); chymostatin (100 \(\mu\)g/ml); antipain (40 \(\mu\)g/ml); and pepstatin (40 \(\mu\)g/ml). Control lane represents nontreated cells. The cells were then lysed in sample buffer and analyzed by immunoblotting with ID-7.2.3 antibodies. The appearance of the 78-kDa cell-associated fragment is partially suppressed in the cells incubated with the anti-proteinase mixture.

To further characterize the relationships between the intact A-CAM and the 78-Kda fragment we have studied the effect of cycloheximide on the relative amounts of the two forms following Ca\(^{2+}\) depletion. The results indicated that the initial rates of both the appearance of the latter and the decline of A-CAM were not significantly altered by cycloheximide. Yet following incubations for 3–24 hr both processes were partially suppressed. This observation is consistent with the possibility that the 78-kDa molecule is indeed derived from A-CAM but the activity or apparent levels of the relevant proteinase are affected by cycloheximide.

To determine whether the cleavage of A-CAM in LCa\(^{2+}\) medium is attributable to a cell-associated proteinase or can be assigned to soluble proteinases present in the culture medium, we collected the media of lens cells maintained in LCa\(^{2+}\) medium for different periods of time and checked their effect on A-CAM integrity when added to fresh lens cell cultures. There was no significant difference between the apparent extents of A-CAM cleavage in cells incubated with either fresh or with 24 hr conditioned LCa\(^{2+}\) medium (not shown). This finding indicates that there is no time-dependent accumulation of such proteinase(s) in the medium. Similarly, varying the fetal calf serum concentration in the LCa\(^{2+}\) medium from 1–10% did not significantly affect the rate of A-CAM cleavage (not shown).

The cellular fate and localization of the 78 kDa fragment formed in LCa\(^{2+}\) medium were studied also by immunofluorescent and immunoelectron microscopy. As previously shown (see Volk and Geiger, 1986a) depletion of Ca\(^{2+}\) ions from lens cell cultures resulted in a rapid disruption of cell junctions leading to a remarkable contraction of the cells which was apparent within 30 sec of exposure to EGTA (see also Kartenbeck et al. 1982; Volberg et al., 1986). However, upon longer periods of incubation in LCa\(^{2+}\) medium, the cells underwent resdepending, though intracellular adherens junctions were apparently not reformed. To compare the distributions of the intact A-CAM and the 78-kDa fragment in untreated and EGTA-treated lens cells, cultures were immunolabeled either with mAb ID-7.2.3 (which reacts with both the intact A-CAM and the 78-kDa fragment) or with antibody CC-11 which binds to the former only and fails to bind to the fragment. The results (Fig. 7) show that, while both antibodies similarly stained the intact A-CAM in junctional sites, antibody ID-7.2.3 yielded uniform surface labeling following 24 hr in LCa\(^{2+}\) medium, and there was, apparently, no staining with antibody CC-11. It is noteworthy that similar labeling was obtained also when the EGTA-treated cells were fixed and labeled without a permeabilization step. Immuno EM labeling of ultrathin frozen sections confirmed these observations at the ultrastructural level. Antibody ID-7.2.3 labeled the junctions between lens cells cultured in NCa\(^{2+}\) medium (Fig. 8). Following the change to LCa\(^{2+}\) medium, positively labeled molecules were sparsely distributed throughout the cell surface (Fig. 8) as well as in submembrane vesicular structures (see insert in Fig. 8D).

Modulation of A-CAM Levels in Chick Somites: Possible Involvement of Proteolysis

In view of the capacity of the two types of anti-A-CAM mAbs (ID 7.2.3 and CC-11) to distinguish intact A-CAM from the cleaved 78-kDa form, we have used the two to examine possible changes in A-CAM in developing chick embryos. We have previously shown (Duband et al., 1988) that A-CAM is expressed in a large number of epithelia in developing chick embryos, including the neuroepithelium, heart, ectodermal placodes, various mesodermal ducts, dorsal root and sympathetic ganglia, and somites. The latter maintain their epithelial features for a short period only, until their ventromedial portion (the sclerotome) dissociates. Dissociation of the sclerotomal epithelium is accompanied by local disap-
Fig. 7. Immunofluorescent labeling of chicken lens cells cultured in normal medium (A and B) or in LCa++ medium for 24 hr (C and D), using monoclonal antibodies ID-7.2.3 (A and C), or CC-11 (B and D). Notice that the two antibodies similarly stain the junction-associated A-CAM in the control cultures, while the residual molecule present in the EGTA-treated cultures (mostly the 78-kDa polypeptide) is uniformly distributed over the cell surface and is reactive with mAbs ID-7.2.3 only. The bar indicates 10 μm.

Appearance of A-CAM/N-cadherin labeling (Duband et al., 1987, 1988).

Immunolabeling of serial sections of 3-day-old chick embryo with antibodies ID-7.2.3 and CC-11 revealed that, at initial stages of sclerotome dissociation, there was a significant difference between the labeling patterns with the two antibodies. This was manifested by an intense staining with mAb 7.2.3 in ventromedial regions of the somites in which staining with mAb CC-11 was weak or absent (Fig. 9). This observation agrees with the possibility that proteolytic cleavage of A-CAM occurs as one of the first events preceeding sclerotome dissociation. It is noteworthy that the sclerotomes of more anterior somites were negative with the two antibodies while the ventromedial region of more posterior somites was extensively labeled with both (Fig. 9, and see Duband et al., 1988). It should be emphasized that in these experiments the intensity of labeling with antibody CC-11 in the latter sites was comparable or higher than that obtained with ID-7.2.3.

We also attempted to examine this possibility directly. We dissected trunk segments of 3-day-old chick embryos containing dissociating somites, and subjected them to an immunoblotting analysis with antibody ID-7.2.3. While in most cases we were able to identify major 78- and 97-kDa-fragments (in addition to a 135-
kDa band), it was difficult to estimate reproducibly the relative amounts of these bands (compared to intact A-CAM) in dissociating versus nondissociating somites, possibly reflecting the transient nature of these fragments of A-CAM (see Discussion).

**DISCUSSION**

Studies in many laboratories have established the tight correlation between the local expression of specific cell adhesion molecules and the acquisition of distinct histotypic and cellular patterns. These correla-
Fig. 9. Immunofluorescent labeling of chick embryo somites with ID-7.2.3 and with CC-11 monoclonal antibodies. Serial sagittal sections show developing somites (A, A') as well as somites located at more rostral positions (B, B' and C, C'). Sections 60-66 hr old of chick embryo (stage 17) were labeled with ID-7.2.3 (which also recognizes the 78-kDa fragment) (A, B, C) or with CC-11 (which is not reactive with the 78-kDa fragment (A', B', C'). The ventral region of the somites is gradually dissociated, and the somites in C, C' are already reorganized into the dermamyotome. Notice that the ventral dissociating region of the somites is labeled strongly with ID-7.2.3 but not with CC-11. The dermamyotome region is strongly labeled with both antibodies. Bar represents 10 μm.
tions between CAM expression and specific morphogene-
tic processes were extensively documented both for
developing embryos (Hatta and Takeichi, 1986; Duband
et al., 1987, 1988 and for review, Edelman 1985, and
Takeichi, 1988) and cultured cells transfected with
cDNA clones encoding different CAMs (Edelman et al.,
1987; Hatta et al., 1988; Mege et al., 1985). Moreover,
these studies indicated that cadherin-mediated inter-
actions may be involved in cell sorting either in culture
or in developing embryonic tissues (Miyatani et al.,

In the present study we present results suggesting
that the activity of A-CAM, a cadherin molecule present
in a multitude of cell types in developing embryos,
may also be down-regulated by proteolysis. We describe
two truncated forms of A-CAM, a 78-kDa, membrane-
bound polypeptide, and a ~97-kDa polypeptide released
into the medium. We further propose that the two
polypeptides were derived from A-CAM by the ac-
tion of endogenous proteinases in low or normal Ca^{2+}
concentrations, respectively. Here we would like to dis-
cuss several aspects of the apparent proteolytic cleav-
age of A-CAM and its possible physiological signifi-
cance. The major questions to be considered include: (a)
To which of the A-CAM domains do the 78- and 97-kDa
polypeptides correspond? (b) What is the evidence that
these polypeptides are indeed derived from A-CAM by
proteolysis? (c) If indeed proteinases are involved in the
formation of the 78- and 97-kDa polypeptides, what are
the origins, cellular distributions and properties of
these enzymes? (d) Does proteolytic cleavage of A-CAM
occur in intact developing tissues and, if so, what might
the functional significance of the cleavage be?

The assignment of the 78- and 97-kDa polypeptides to
the C-terminal and N-terminal portions of A-CAM, re-
spectively, is based mainly on the known primary
structure and submolecular domains of N-cadherin/A-
CAM (see Hatta et al., 1988). It is supported by the tight
association of the 78-kDa polypeptide with the plasma
membrane, the appearance of the 97-kDa fragment in
the medium and the reactivities of the two polypeptides
with several monoclonal antibodies with defined speci-
ficities. The indication that the 78-kDa polypeptide
formed in LCa^{2+} medium contains the intact C-terminal
cytoplasmic domain of A-CAM, the transmembrane re-
region and part of the extracellular domain is supported
by the reactivity of this polypeptide with both the C-
terminus-reactive antibody R-156 and with antibody
ID-7.2.3 which reacts with the extracellular domain of
A-CAM, proximal to the transmembrane sequences (see
Volk and Geiger, 1984, 1986a). Consequently, the 78-kDa
polypeptide is not reactive with several A-CAM-specific
monoclonal antibodies which bind to the N-terminal
region of the molecule. The 97-kDa fragment released
from the cell surface to the medium reacts with the
latter antibodies as well as with mAb ID-7.2.3, but not
with antibody R-156, suggesting that it corresponds to
the extracellular moiety of the A-CAM molecule.

We propose here that both the 78- and 97-kDa forms
of A-CAM are derived from the intact molecule by en-
dogenous proteinases. This suggestion is strongly sup-
portsed by several lines of evidence: (a) the 78-kDa poly-
peptide seems to be essentially identical to the major
proteolytic product formed following trypsin-EGTA
treatment (Volk and Geiger, 1986a). Interestingly, a
similar cleavage of A-CAM was also noticed following
addition of other proteinases including V-8 and bromo-
lain to EGTA-treated cells (Volk and Geiger, unpub-
lished results). It is noteworthy that longer incubation
of cells with trypsin-EGTA, resulted in the gradual dis-
appearance of the 78-kDa tryptic fragments and the
appearance of 60- and 46-kDa bands that also react with
mAbs ID 7.2.3. This could be a result of further cleavage
of the 78-kDa molecule by trypsin or by some endoge-
nous proteinases. According to the results presented
here, the latter possibility is more plausible in view of
the failure of R-156 antibody to react with these two
fragments. Since the exogenously added trypsin does
not penetrate into the cell, we suggest that cytoplasmic
proteinase(s) cleave the C-terminal region of the 78-
kDa fragment or parts of it following treatment with
trypsin-EGTA. (b) the progressive accumulation of the
78-kDa polypeptide on the surface of EGTA-treated
cells closely parallels the decline of intact A-CAM in the
treated cells, suggesting that they maintain product-
precursor relationships; (c) the appearance of the 78-
kDa polypeptide following LCa^{2+} treatment could be
partially inhibited by a cocktail of proteinase inhibi-
tors. (d) The release of the 97-kDa molecule into the
medium, is suppressed by a short pretreatment of the
cultured lens cells with trypsin-EGTA (but not with
either trypsin or EGTA alone), suggesting that this
fragment is indeed derived from a membrane-bound,
trypsin-EGTA-sensitive, precursor.

The mode of involvement of Ca^{2+} ions in the cleavage
of A-CAM into the 78-kDa fragment deserves some fur-
ther discussion and clarification. In a previous study
dealing with trypptic cleavage of A-CAM, we raised the
possibility that, in the presence of normal Ca^{2+} concen-
trations, adherens junctions remain intact and thus
A-CAM may be inaccessible to trypsin (Volk and Geiger,
1986a). This interpretation for the role of Ca^{2+}
in the protection of A-CAM from proteolysis was
adopted since we have found that cleavage of A-CAM
occurred even when trypsin was added to cells in Ca^{2+}-
containing medium, provided that the cells were in-
ubated with EGTA for a few minutes immediately prior
to the addition of trypsin (Fig. 1; and Volk and Geiger,
tion of this A-CAM-related molecule: (a) proteolysis, (b) 
may involve Ca\(^{2+}\)-dependent changes in the conformation 
of A-CAM and/or in its state of aggregation, which 
may affect both the biological activity of the molecule 
and its sensitivity to proteolysis. Studies addressing 
this aspect are now in progress.

Unlike the formation and accumulation of the 78-kDa 
polypeptide, the formation and release of the 97-kDa 
molecule were not affected by Ca\(^{2+}\) ions. Initially, we 
considered two alternative mechanisms for the forma-
tion of this A-CAM-related molecule: (a) proteolysis, (b) 
the presence of a molecular variant of A-CAM, encoded 
by a different gene or alternative spliced mRNA. How-
ever, the former view seems more likely in as much as 
only one gene and a single message encoding N-cad-
herin has been detected (Hatta et al., 1986a). However, 
we have shown that, following cleavage of 
A-CAM by trypsin in the presence of EGTA, the levels 
of the 97-kDa polypeptide subsequently released into the 
medium declined dramatically, suggesting that the 
latter is indeed derived from a trypsin-EGTA sensitive 
A-CAM at the cell surface.

Interestingly, the two modes of cleavage of A-CAM 
by endogenous proteinases as described here appear to 
be related to the two types of trypsin digestion described 
for several cadherins. Trypsin treatment of cells in the 
presence of Ca\(^{2+}\) results in the release from the cell 
surface of \(~80- to 82-kDa\) fragment of uvomorulin 
(Hyafil et al., 1980; Peyrieras et al., 1983), cell-CAM 
120/80 (Damsky et al., 1983), chicken L-CAM (Cun-
ningham et al., 1984), and E-cadherin (Yoshida and Ta-
keichi, 1982). Tryptic digestion of these molecules in the 
absence of Ca\(^{2+}\) ions results in the formation of frag-
ments that were undetectable with the antibodies used 
in these studies, perhaps because the antibodies recog-
nize the N-terminal region of the molecule.

Cell surface proteinases have been described in a 
variety of cellular systems (see i.e., Zucker-Franklin et al., 
1981; Couch and Strittmatter, 1983; Sloane et al., 1986; 
Tanaka et al., 1986; Chen and Chen, 1987). These en-
zymes have been primarily implicated in cell invasive-
ness through the extracellular matrix. Furthermore, in 
some cases, they may cleave specific membrane-assoc-
iated molecules. Such processes had been recently re-
ported for syndecan, a cell surface proteoglycan of epi-
thelial cells, that binds to various ECM molecules (Jal-
kanen et al., 1987; Saunders et al., 1989). The ectodomain 
of syndecan, which contains the glycosaminoglycan 
chains, can be cleaved by trypsin or by endogenous pro-
teinase and released into the medium.

Does the proteolytic cleavage of A-CAM bear any 
physiological significance? Can similar proteolytic pro-
cesses be detected in developing tissues undergoing 
modification? Such possibility appeared attractive in 
view of our previous results (Duband et al., 1987, 1988) 
which indicated that local disappearance of A-CAM/N-
cadherin from a variety of embryonic tissues is closely 
correlated with deep epithelialization, e.g., during neural 
crest or somite development. In the latter system, 
A-CAM/N-cadherin is initially highly expressed 
throughout the somitic epithelium, but, as soon as the 
epithelium is at its most differentiated into sclerome and dermamy-
tome, the expression of this cadherin and its mRNA in 
the scleromere rapidly decline (Duband et al., 1988; 
Geffen-Constant and Geiger, unpublished results). 
However, a mechanism whereby the turnover of 
A-CAM is locally increased seemed an equally attrac-
tive mechanism for a rapid modulation of cell–cell 
junctions.

To examine whether specific degradation of A-CAM 
takes place in differentiating somites, we have initially 
tried to identify breakdown products in dissociating so-
mites by microdissection of the relevant tissue seg-
ments followed by immunoblotting with mAb 1D-7 23. 
In these experiments we have indeed detected both 78-
and 97-kDa immunoreactive bands, yet the amounts of 
these components relative to intact A-CAM varied from 
one experiment to the next. Since we could not exclude 
the possibility that cleavage of A-CAM occurred (at 
least partly) at the time of or following the dissection of 
the relevant tissue segments, we have used an alterna-
tive, immunocytochemical approach, based on the ca-
pacity of different mAbs to distinguish the intact 
A-CAM from its various proteolytic fragments. Immu-
nofluorescent labeling of serial sections by such a pair 
of antibodies showed significant differences, at the 
onset of sclerome dissociation. We observed signif-
ificant labeling with the antibody that binds to the 78-kDa 
fragment in scleromeral regions, while the intact 
A-CAM was already undetectable by immunofluores-
cence. While the basis for the apparent differences in 
the immunolabeling is not clear, the findings described 
here agrees with the notion that A-CAM is cleaved in 
vivo and that the cleavage is spatial-temporally related 
to the down-regulation of A-CAM expression. Using the 
immunocytochemical analysis described here, and in 
view of the small amounts of material available for
direct molecular analysis, we could not determine the exact molecular nature of the major cleavage product of A-CAM in developing somites. Nevertheless, it apparently exhibits an overall immunoechemical reactivity similar to that of the 78-kDa peptide. Further biochemical analysis will be required to substantiate this suggestion.

The findings described here raise a number of questions concerning alternative and complementary mechanisms which are involved in the physiological modulation of cell contacts. Is a proteolytic cleavage of A-CAM in vivo triggered by local modulation of intercellular Ca\(^{2+}\) concentrations? Is the proteolytic enzyme(s) involved in A-CAM cleavage constitutively expressed or is it expression spatially and temporally regulated during development? Can the released 97-kDa fragment or the released peptides generated following cleavage of A-CAM in LCa\(^{2+}\) medium bind to surface-associated A-CAM and inhibit the formation of intercellular junctions? It has been previously shown that the isolated ectodomain of uvomorulin (Cell CAM 120/80) is capable of inhibiting epithelial cell–cell adhesions (Wheelock et al., 1987). We believe that further studies on the involvement of proteinases will provide answers for these questions and will shed new light on the various alternative mechanisms involved in the modulation of intercellular junctions.

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