DEVELOPMENTAL BIOLOGY 139, 314-326 (1990)

Cleavage of A-CAM by Endogenous Proteinases in Cultured Lens Cells and in Developing Chick Embryos

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Accepted January 16, 1990

We describe two truncated forms of A-CAM (N-cadherin) and present evidence suggesting that both forms are proteolytically derived from the intact A-CAM molecule. The first is a membrane-bound fragment of A-CAM displaying an apparent molecular weight of 78 kDa. This polypeptide, containing the C-terminal portion of the protein, may be generated in cultured chicken lens cells, either by a short treatment with trypsin-EGTA, or by endogenous proteinase(s) during incubation in low Ca^{2+} medium. Immunofluorescent labeling of normal and EGTA-treated cells indicated that the 78-kDa fragment is uniformly distributed over the cell surface. Moreover, staining of developing chick embryos with pairs of antibodies which distinguish the 78-kDa fragment from intact A-CAM indicated that, at early stages of sclerotome dissociation in developing somites, a truncated derivative of the molecule is generated. The second truncated form of A-CAM is a 97-kDa polypeptide which is constitutively released by cultured lens cells into the culture medium in the presence of normal medium. We present evidence that the 97-kDa molecule is proteolytically derived from A-CAM by the action of an endogenous proteinase. We discuss possible mechanisms leading to the formation of these two truncated derivatives and their possible involvement in the physiological modulation of A-CAM-mediated interactions.

INTRODUCTION

A-CAM[2] is a Ca^{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, α-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 *in situ* hybridization (ffrench-Constant and B. Geiger, unpublished data) indicate that

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[2] Abbreviations used: A-CAM, adherens junction specific cell adhesion molecule; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LCa^{2+} and NCa^{2+}, low (0.3 m*M*) and normal Ca^{2+} (1.8 m*M*)-containing medium; PMSF, phenylmethylsulfonyl fluoride.

0012-1606/90 $3.00

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 proteinase(s). 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^{2+}, results in a constitutive release into the culture medium of a 97-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 m*M* Tris-Histidine buffer, 20 m*M* 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 m*M* and the solution was further homogenized for 5 min in a loose Dounce homogenizer. $CaCl_2$ was then added up to 0.1 m*M*, the extract was filtered through cheese cloth and centrifuged in a refrigerated Sorvall SS 34 rotor at 3000*g* for 10 min. The pellet was washed three times with buffer A, suspended in buffer B (10 m*M* 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 m*M* NaCl, and 50 m*M* 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 m*M* 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 $F(ab')_2$ 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_4OH. 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 m*M* 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 m*M* Tris-glycine buffer, pH 8.6, containing 1 m*M* $MgCl_2$. 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 ^{125}I-labeled goat anti-mouse $F(ab')_2$. 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 × 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 m*M* 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 (DY-DYLNDWGPRFKKLADMYGGGDD; 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 (Brandtzaeg, 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% $NaBH_4$, washed extensively with H_2O, 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 N_2 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 Ca^{2+} Ions

As previously described, the addition of trypsin in serum-free Ca^{2+}-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 Ca^{2+} ions were depleted from the culture medium by EGTA (Low Ca^{2+}, <0.3 m*M* (LCa^{2+})), 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

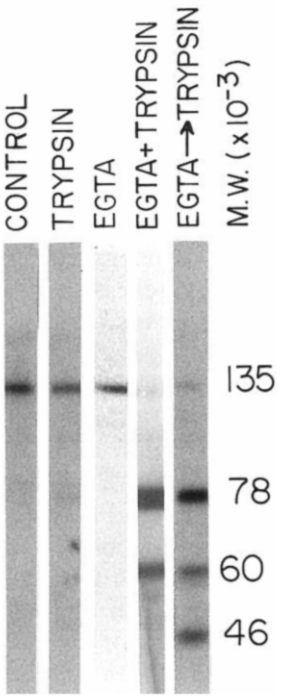

FIG. 1. Immunoblotting analysis of cultured chicken lens cells using ID-7.2.3 (anti A-CAM) monoclonal antibodies. The cells in 35-mm culture dishes were treated for 10 min with trypsin (100 μg/ml), 5 m*M* EGTA, or both together (trypsin + EGTA, all in serum-free DMEM). Alternatively, cells were incubated with 5 m*M* EGTA for 10 min and then exposed to trypsin in normal medium for additional 10 min. Note that A-CAM was not significantly affected by EGTA or trypsin alone but was readily cleaved when the two were applied either together or when trypsin was added in Ca^{2+}-containing medium immediately following pretreatment with EGTA. The major cleavage products are bands corresponding to 78-, 60-, and 46-kDa, all of which are recognized by antibody ID-7.2.3.

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 m*M* 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

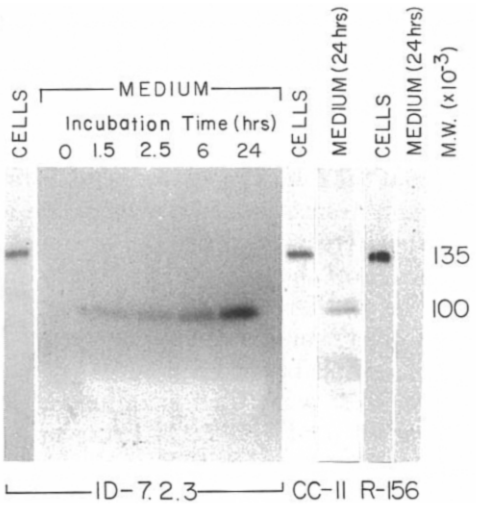

FIG. 3. Distinct immunological reactivities of the various anti A-CAM antibodies with the released 97-kDa fragment of A-CAM. The medium in which lens cells were incubated for increasing time periods (0, 1.5, 2.5, 6, and 24 hr) was collected, boiled in sample buffer, and examined by immunoblotting with ID-7.2.3. Samples of the 24-hr conditioned medium were also analyzed for reactivity with monoclonal antibody CC-11 and with R-156 (anticytoplasmic domain of A-CAM). The lens cells were lysed and analyzed as above with ID-7.2.3, CC-11, and R-156 antibodies. Notice that the amount of the 97-kDa polypeptide increases with incubation time. The CC-11 antibody also recognizes the 97-kDa form of A-CAM, while R-156 does not react with it.

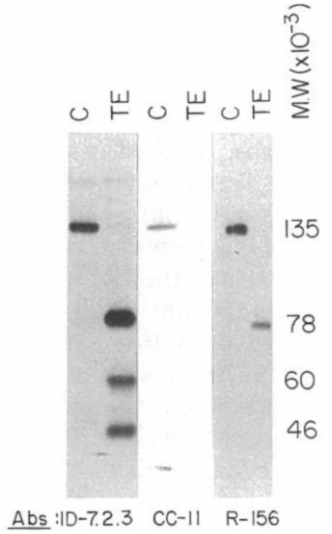

FIG. 2. Specificity of anti A-CAM antibodies used in this study. Untreated control lens cells (C) or cells pretreated for 10 min with trypsin and EGTA (TE) were examined by immunoblotting with various A-CAM-specific antibodies, including monoclonal antibodies ID-7.2.3 and CC-11 as well as rabbit antibodies directed against the C-terminal 24 amino acids (R-156). All antibodies bind to the intact A-CAM (~135 kDa). The cell-bound cleavage product of 78-kDa is recognized by antibodies ID-7.2.3 and R-156 but not by CC-11. The 60- and 46-kDa fragments lost their reactivity with antibody R-156, suggesting that the C-terminal region was also truncated probably by a cytoplasmic proteinase.

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 NCa^{2+} 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 Ca^{2+} Medium

As shown above, surface-associated A-CAM was readily cleaved by trypsin either in LCa^{2+} medium or

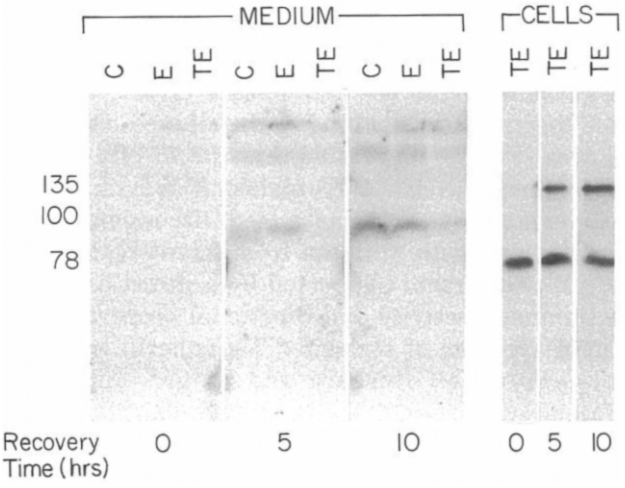

FIG. 4. The effect of EGTA–trypsin pretreatment on the subsequent release of the 97-kDa, A-CAM-related polypeptide. Subconfluent cultured lens cells in 35-mm dish were incubated in low Ca^{2+} medium (3 m*M* EGTA) (E), or in the same low Ca^{2+} medium containing 0.1 mg/ml trypsin for 5 min. The cells 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.

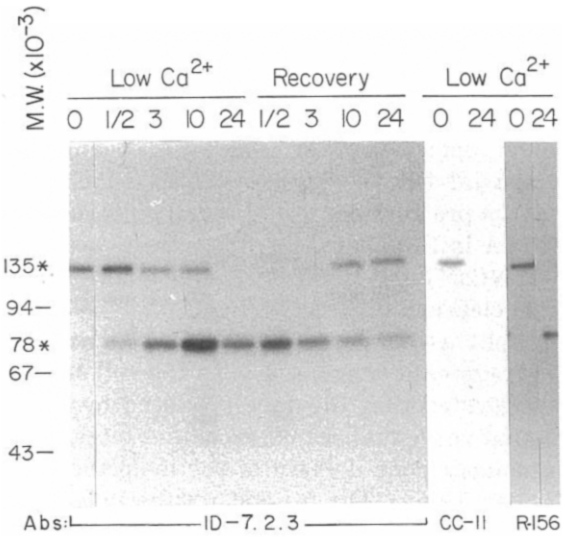

FIG. 5. Cleavage of A-CAM in low Ca^{2+} medium. Cultured lens cells (subconfluent culture in 35-mm dishes) were incubated as indicated (0–24 hr) in 1 ml DMEM containing 5 m*M* EGTA, lysed in sample buffer and examined by immunoblotting using antibodies ID-7.2.3, CC-11, or R-156. In addition, cells preincubated for 24 hr in LCa^{2+} medium were switched to NCa^{2+} levels (recovery) for the indicated periods. Notice that in low Ca^{2+} 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).

when applied immediately following EGTA treatment (Fig. 1), but was not rapidly altered by depletion of Ca^{2+} ions alone. However, prolonged incubation of cultured lens cells in LCa^{2+} 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 LCa^{2+} medium and the intact A-CAM essentially disappeared following 24 hr of incubation (Fig. 5). Switch of EGTA-treated cultures into NCa^{2+} 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), bestatinal (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.

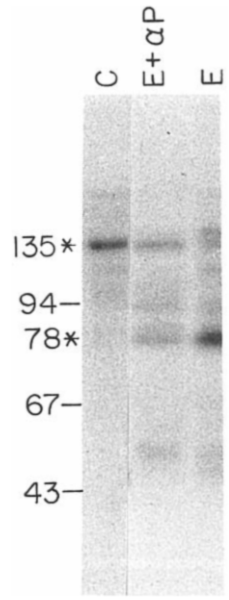

FIG. 6. Partial inhibition of A-CAM proteolysis in low Ca^{2+} medium by a mixture of proteinase inhibitors. Cultured lens cells were incubated in low Ca^{2+} medium for 20 hr (E). One hour prior to addition of EGTA, a mixture of proteinase inhibitors was added (E + αP). This mixture contained: leupeptin (100 μg/ml); elastinal (100 μg/ml); bestatinal (100 μg/ml); chymostatin (100 μg/ml); antipain (40 μg/ml); and pepstatin (40 μg/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 respreading, 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-

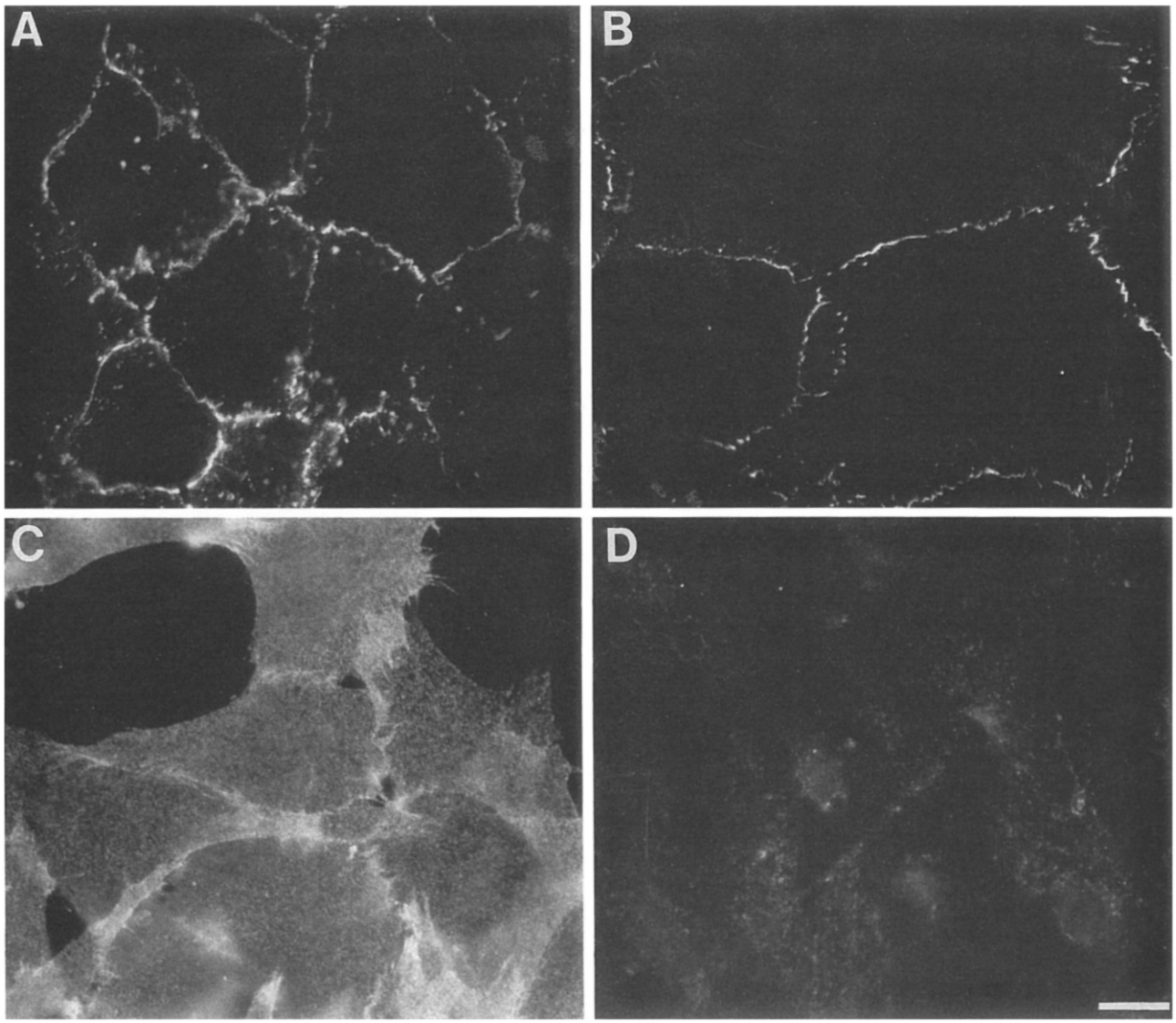

FIG. 7. Immunofluorescent labeling of chicken lens cells cultured in normal medium (A and B) or in LCa^{2+} medium for 24 hr (C and D), using monoclonal antibodies ID-7.2.3 (A and C), or CC-11 (B and D). Notice that the two antibodies similarly stain the junction-associated A-CAM in the control cultures, 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.

pearance 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-

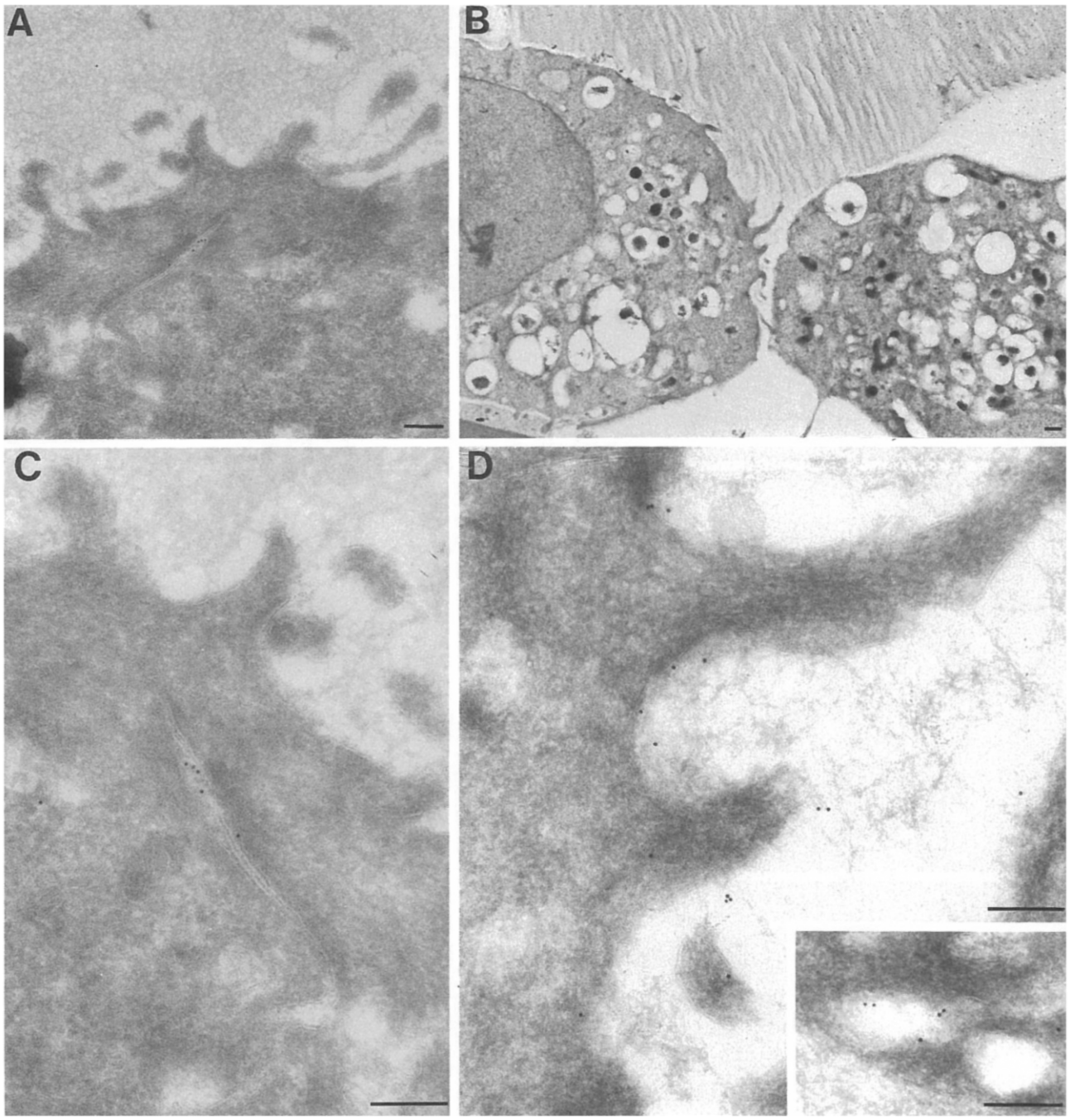

FIG. 8. Immunoelectron microscopic labeling of A-CAM in lens cells cultured in NCa^{2+}-containing medium (A and C) or in LCa^{2+} medium (B and D). The distribution of the molecule is demonstrated by immunogold labeling of ultrathin frozen sections and is shown at low- (A and B) and high- (C and D) power magnifications. Notice the overall effect of EGTA-treatment on cell shape and cell-cell junctions. The immunogold label is restricted mainly to the junctions in control cells (C) while in the EGTA-treated cells the labeling is detected in scattered patches, or in submembrane vesicles or membrane foldings (see inset in D). The bars indicate 0.2 μm.

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-

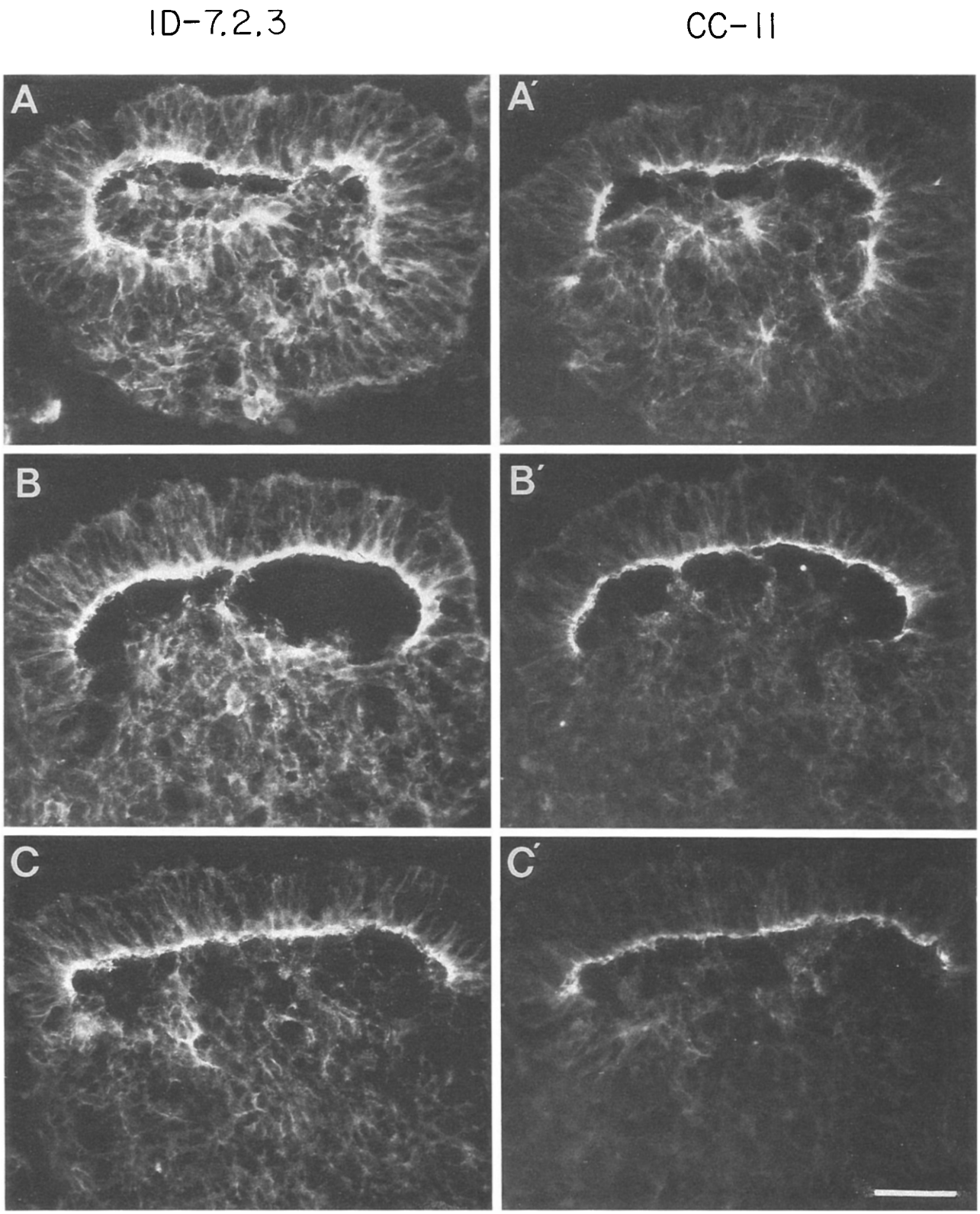

FIG. 9. Immunofluorescent labeling of chick embryo somites with ID-7.2.3 and with CC-11 monoclonal antibodies. Serial sagital sections show developing somites (A, A') as well as somites located at more rostral positions (B, B' and C, C'). Sections 60–66 hr old of chick embryo (stage 17) were labeled 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 morphogenetic 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.*, 1988). Moreover, these studies indicated that cadherin-mediated interactions may be involved in cell sorting either in culture or in developing embryonic tissues (Miyatani *et al.*, 1989; Hirai *et al.*, 1989).

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 an ~97-kDa polypeptide released into the medium. We further propose that the two polypeptides were derived from A-CAM by the action of endogenous proteinases in low or normal Ca^{2+} concentrations, respectively. Here we would like to discuss several aspects of the apparent proteolytic cleavage of A-CAM and its possible physiological significance. 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, respectively, 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 specificities. The indication that the 78-kDa polypeptide formed in LCa^{2+} medium contains the intact C-terminal cytoplasmic domain of A-CAM, the transmembrane 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 endogenous proteinases. This suggestion is strongly supported by several lines of evidence: (a) the 78-kDa polypeptide 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 bromelain to EGTA-treated cells (Volk and Geiger, unpublished results). It is noteworthy that longer incubation of cells with trypsin–EGTA, resulted in the gradual disappearance 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 endogenous 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 inhibitors. (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 further discussion and clarification. In a previous study dealing with tryptic cleavage of A-CAM, we raised the possibility that, in the presence of normal Ca^{2+} concentrations, 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 incubated with EGTA for a few minutes immediately prior to the addition of trypsin (Fig. 1; and Volk and Geiger,

1986a). However, in a recent series of studies (Volberg and Geiger, unpublished observations), we have noticed that trypsin resistance of A-CAM was regained when EGTA-treated cells were incubated in normal (Ca^{2+}-containing) medium for 10–30 min prior to the addition of trypsin. Notably the reformation of new cell–cell junctions was a much slower process, apparent only following 2 hr or more of recovery. These findings suggest that the protection of A-CAM from proteolysis by trypsin (and possibly also by the endogenous proteinase(s) discussed here) cannot be attributed merely to inaccessibility of the enzyme to the junctional cleft but 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 formation 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. However, the former view seems more likely in as much as only one gene and a single message encoding N-cadherin/A-CAM has been detected (Hatta *et al.*, 1988). Moreover, 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 tryptic 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 (Cunningham *et al.*, 1984), and E-cadherin (Yoshida and Takeichi, 1982). Tryptic digestion of these molecules in the absence of Ca^{2+} ions results in the formation of fragments that were undetectable with the antibodies used in these studies, perhaps because the antibodies recognize 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 enzymes have been primarily implicated in cell invasiveness through the extracellular matrix. Furthermore, in some cases, they may cleave specific membrane-associated molecules. Such processes had been recently reported for syndecan, a cell surface proteoglycan of epithelial cells, that binds to various ECM molecules (Jalkanen *et al.*, 1987; Saunders *et al.*, 1989). The ectodomain of syndecan, which contains the glycosaminoglycan chains, can be cleaved by trypsin or by endogenous proteinase and released into the medium.

Does the proteolytic cleavage of A-CAM bear any physiological significance? Can similar proteolytic processes be detected in developing tissues undergoing modulation? 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 deepithelialization, 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 somite differentiates into sclerotome and dermamyotome, the expression of this cadherin and its mRNA in the sclerotome rapidly decline (Duband *et al.*, 1988; ffrench-Constant and Geiger, unpublished results). However, a mechanism whereby the turnover of A-CAM is locally increased seemed an equally attractive 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 somites by microdissection of the relevant tissue segments followed by immunoblotting with mAb ID-7.2.3. 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 alternative, immunocytochemical approach, based on the capacity of different mAbs to distinguish the intact A-CAM from its various proteolytic fragments. Immunofluorescent labeling of serial sections by such a pair of antibodies showed significant differences, at the onset of sclerotome dissociation. We observed significant labeling with the antibody that binds to the 78-kDa fragment in sclerotomal regions, where the intact A-CAM was already undetectable by immunofluorescence. While the basis for the apparent differences in the immunolabeling is not clear, the finding described here agrees with the notion that A-CAM is cleaved *in vivo* and that the cleavage is spatiotemporally 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 immunochemical 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 its 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.

This study was supported by the Fusfeld Foundation and the Revson research grant awarded by the Israel National Academy of Sciences and by the Fors. We express our gratitude to Robert Bloch for critically reading the manuscript and for his enlighting comments. B.G. is the E. Neter Professor for Cell and Tumor Biology. T. Volk's present address is: Molecular Biology Institute, UCLA, 405 Hilgard Avenue, Los Angeles, California 90024-1570, USA.

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