A-CAM: A 135-kD Receptor of Intercellular Adherens Junctions. II. Antibody-mediated Modulation of Junction Formation

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Abstract. Intercellular adherens junctions between cultured lens epithelial cells are highly Ca^{2+}-dependent and are readily dissociated upon chelation of extracellular Ca^{2+} ions. Addition of Ca^{2+} to EGTA-treated cells results in the recovery of cell–cell junctions including the reorganization of adherens junction–specific cell adhesion molecule (A-CAM), vinculin, and actin (Volk, T., and B. Geiger, 1986, *J. Cell Biol.*, 103:000–000). Incubation of cells during the recovery phase with Fab' fragments of anti-A-CAM specifically inhibited the re-formation of cell–cell adherens junctions. This inhibition was accompanied by remarkable changes in microfilament organization manifested by an apparent deterioration of stress fibers and the appearance of fragmented actin bundles throughout the cytoplasm. Incubation of EGTA-dissociated cells with intact divalent anti-A-CAM antibodies in normal medium had no apparent inhibitory effect on junction formation and did not affect the assembly of actin microfilament bundles. Moreover, adherens junctions formed in the presence of the divalent antibodies became essentially Ca^{2+}-independent, suggesting that cell–cell adhesion between them was primarily mediated by the antibodies. These studies suggest that A-CAM participates in intercellular adhesion in adherens-type junctions and point to its involvement in microfilament bundle assembly.

STUDIES on the molecular architecture of adherens-type junctions have suggested that these cellular contacts consist of three major structural domains. These include a membrane domain with specific "contact receptors," a membrane-bound cytoplasmic plaque, and bundles of actin filaments connected to it (20–23). Our previous studies have established that adherens junctions are molecularly heterogeneous with respect to some of their plaque and membrane-associated components. This was manifested by the presence of the junctional-plaque protein, talin, in cell matrix contacts only and the exclusive association of the 135-kD protein, namely the adherens junction–specific cell adhesion molecule (A-CAM),[1] with intercellular junctions (24). In the preceding paper (40) we have provided evidence that A-CAM is a specific junctional surface glycoprotein. We would like to show here that A-CAM participates in intercellular adhesion and that antibody-mediated inhibition of these interactions prevents junction formation and leads to a remarkable deterioration of the cytoplasmic microfilament system.

"Cell adhesion molecules" or "contact receptors" have been the focus of much attention over the last few years. Studies in several laboratories have established the existence of a family of surface proteins that may specifically mediate the attachment of cells to cellular or noncellular surfaces (see 5, 10, 13, 16, 29, 31, 36, 41). It has been shown that such molecules may vary in their cell type–specific expression, in the nature of the surfaces to which they bind, and in their requirement for extracellular Ca^{2+} ions (11, 14, 35). The most common methodology used in such studies was based on the use of poly- or monoclonal antibodies, which can perturb cell contact formation in a large variety of experimental systems. It is not surprising, therefore, that several laboratories have come forward with a variety of components whose molecular interrelationships were often unclear. In some cases a variety of names have been proposed for the same molecule. The protein uvomorulin, for example, which was initially identified as cell adhesion molecule in embryonal carcinoma (29), has also been found in other systems where it was designated L-CAM (16), E-Cadherin (41), cell-CAM 80/120 (10), Arc-1 antigen (5), etc. The various cell adhesion molecules so far identified were primarily defined according to the tissues or cells in which they are expressed (11, 14, 27) or to the mode of interaction that they mediate (calcium dependence, for example). Only rarely was the fine subcellular distribution of the molecule determined. Recently uvomorulin was identified as a constituent of intestinal zonula adherens (6).

Our previous studies have established the presence of A-CAM at the junctional membrane of adherens junctions in cardiac muscle, lens, etc., as well as in cultured cells derived from these and other tissues (39). In this study we have investigated the possible involvement of A-CAM in intercellular adhesion in general and formation of adherens-type junc-

1. *Abbreviation used in this paper*: A-CAM, adherens junction–specific cell adhesion molecule.

tions in particular. We show here that monovalent Fab' fragments of anti-A-CAM antibody can inhibit the formation of intercellular junctions in cultured chick lens cells. Moreover the microfilament network of these Fab'-treated cells undergoes remarkable deterioration, in line with our previous proposal that adherens junctions are organizing centers for the assembly of microfilament system (4, 20, 21, 22). Moreover, we show that intact, divalent antibody does not inhibit junction formation and even render the junction Ca^{2+}-independent. The significance of A-CAM for cell–cell interactions is discussed as well as the molecular relationships between this protein and other cell adhesion molecules.

Materials and Methods

Immunofluorescent Labeling

Immunofluorescent labeling of cultured cells was carried out as described in the preceding article (40). Double immunofluorescent labeling was carried out as described by Geiger and Singer (18).

Immunochemical Reagents

The antibodies used in this study were as follows: Anti-vinculin was prepared in rabbits and affinity purified as described (17, 19). Labeling with anti-A-CAM was carried out using the supernatant of the hybridoma ID-7.2.3 (39). Actin was labeled with either rhodamine-phalloidin (kindly supplied by Dr. H. Faulstich, Max Planck Institute, Heidelberg, FRG) or NBD phallacidin (Molecular Probes, Inc., Junction City, OR). As secondary antibody reagents we have used goat anti-rabbit Ig and goat anti-mouse $F(ab')_2$, both affinity purified. These antibodies were coupled to rhodamine-lissamine sulfonyl chloride or to dichlorotriazinyl amino fluorescein as previously described (3, 7, 18).

Immunoelectron Microscopic Labeling

Lens cells were cultured on 35-mm tissue cultured dishes (No. 3001, Falcon Labware, Oxnard, CA), fixed with 3% paraformaldehyde in PBS for 30 min, and incubated with anti-A-CAM (undiluted culture supernatant). The cells were washed in PBS and further labeled with affinity-purified goat anti-mouse Ig antibodies coupled to 5-nm gold particles (Janssen Pharmaceutica, Beerse, Belgium). After labeling, the cultures were postfixed in 2% glutaraldehyde and processed further for thin sectioning as previously described (39). Sections were stained with uranyl acetate and lead citrate and examined in the Philips EM 410 at 80 kV.

Preparation and Purification of Fab' Fragments from the A-CAM Antibodies

A-CAM antibodies, in a form of ascities fluid, were dialyzed against 10 mM phosphate buffer pH 8.0 and applied to Sepharose 4B bound to protein A (Pharmacia, Sweden). The column was washed with the buffer and elution of antibodies carried out using 0.1 M acetate buffers adjusted to either pH 6.0, 4.5, or 3.0. The eluted antibodies were examined for their anti-A-CAM activity and polypeptide composition. The monoclonal anti-A-CAM (ID-7.2.3) antibodies were recovered in the pH 4.5 eluate. The purified IgG was digested with pepsin, largely according to Nisonoff (30). It was however essential to define the optimal pH for cleavage of the particular monoclonal antibody used. The pH of the ID-7.2.3 antibody solution was therefore titrated to 3.9 with 4 M acetate buffer pH 3.0. Pepsin (1 mg/25 mg of antibody) was added to the IgG and the solution incubated for 16 h at 37°C. The reaction was terminated by elevating the pH to 8.0 with 2 M Tris. Reduction of the $F(ab')_2$ was performed using 10 mM dithiothreitol for 1 h followed by alkylation with 10 mM iodoacetamide. The Fab' fragments were then dialyzed against PBS.

Treatment of Cultured Lens Cells with Anti-A-CAM

Cultured lens cells were grown on coverslips to near confluence and then treated with 5 mM EGTA for 10 min. The coverslips were then rinsed with fresh medium, transferred to new culture dishes, and incubated with the antibodies. The Fab' fragments or intact antibodies, diluted 1:1 with Dulbecco's modified Eagle's medium (DME) to a final concentration of 0.5 mg/ml, were added. After short incubation (20 min) at room temperature, the cells were then incubated at 37°C for several hours as indicated.

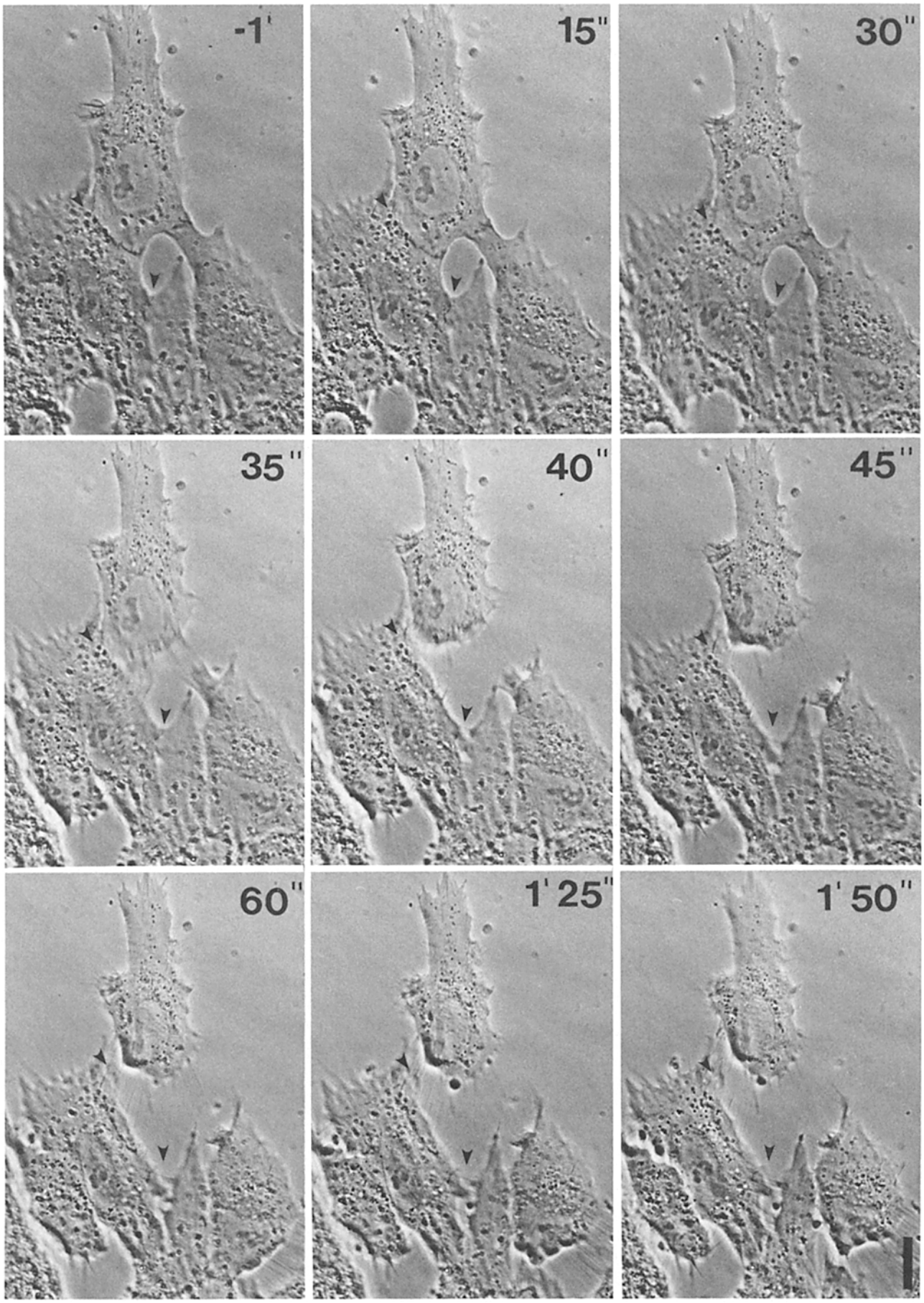
Results

Effect of Ca^{2+} Depletion on Adherens Junctions of Cultured Lens Cells

Cultured chick lens cells form extensive intercellular junctions that exhibit remarkable dependence on the level of extracellular Ca^{2+} ions. Addition of 5 mM EGTA to the culture medium induced a rapid and nearly synchronous cleavage of cell–cell contacts. As seen in Fig. 1 the effect was first apparent ~35 s after addition of EGTA and was essentially complete within less than an additional 20–30 s. Careful examination of treated cultures with phase-contrast microscopy often reveals fine fibers that were attached to the retracting surfaces. These could be identified as fibronectin-containing fibers by immunofluorescent labeling as shown in Fig. 2. The fibronectin bundles were usually detected basally to the intercellular adherens junction as revealed by transmission electron microscopy (see arrows in Fig. 3 *C*).

Electron microscopic examination of cultured lens cells showed that the subapical contact area was of the adherens type, displaying tight association with microfilament bundles (Fig. 3 *A*). Evidently, unlike the lateral association with the membrane of junctional actin bundles in the polarized epithelium of the intestine, the junctional filaments in cultured lens epithelial cells as visualized by electron microscopy and by phalloidin labeling were apparently associated with the membrane in an "end on" configuration. Examination of EGTA-treated cultures at the electron microscopic level did not reveal intact adherens junctions. Moreover due to the rapid retraction of the cells from each other we could rarely identify with certainty the two membrane segments, which were formerly associated with each other in the intact adherens junctions. However examination of cases in which the two retracting cells still retained spatial proximity (Fig. 3 *B*) indicated that the junctional actin filament bundles were still associated with the plasma membrane. This association was apparently retained even in the more advanced stages of cell retraction as shown in Fig. 3 *C*. The identification of the detached junctional membrane, as described above, was mainly based on the presence of actin filament bundles at the cytoplasmic faces of the plasma membrane. This was further corroborated by indirect immunoelectron microscopy using anti-A-CAM antibodies followed by gold-conjugated secondary antibodies (Fig. 3 *E*). The immunogold particles were concentrated on the microfilament-bound junctional membrane of freshly dissociated cells. It should be added that cultured lens cells often form gap junctions with each other (see

Figure 1. Phase-contrast micrographs of living cultured lens epithelial cells treated with 5 mM EGTA for different periods of time, as indicated at the upper right corners. The arrowheads point to sites of definitive cell–cell junctions that are cleaved after Ca^{2+} removal. Notice that the three frames in the middle were taken at 5-s intervals. The first frame (*–1*) was taken 1 min before application of EGTA. Bar, 10 μm.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60"
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1'50"

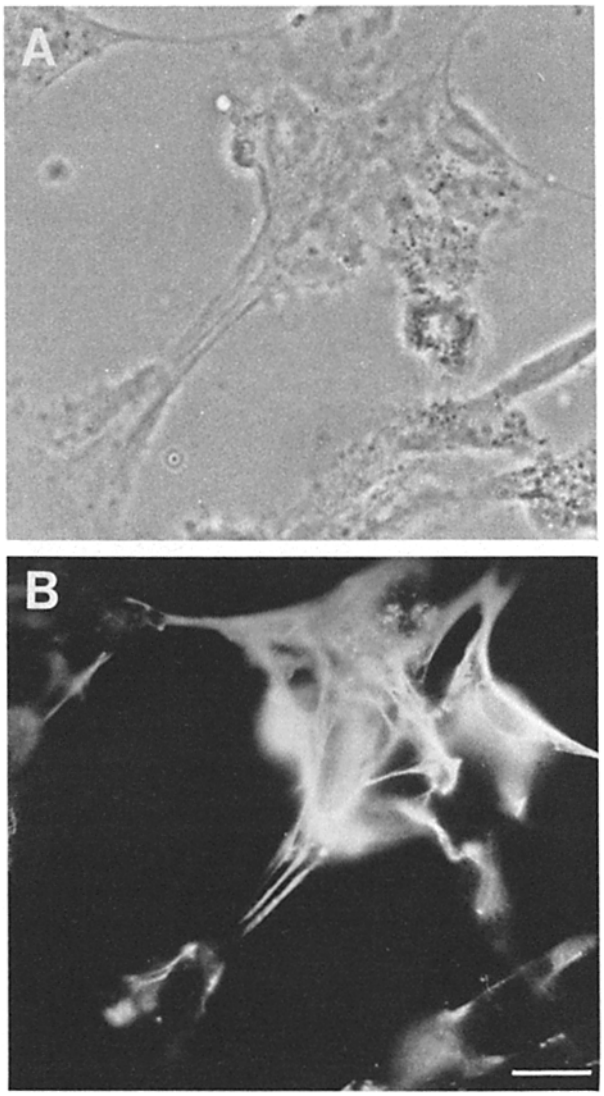

Figure 2. Phase-contrast (*A*) and the corresponding immunofluorescent labeling for fibronectin (*B*) of cultured lens cells treated with EGTA (5 mM) for 10 min. Notice the positive labeling of extracellular fibers attached to the surfaces of the contracting cells. The labeling was carried out on formaldehye-fixed, nonpermeabilized cells. Bar, 10 μm.

references 12 and 34). Examination of EGTA-treated cultures has shown that in contrast to adherens junctions, gap junctions were relatively stable and did not readily dissociate upon depletion of extracellular Ca^{2+} (Fig. 3 *D*).

Spatial Relationships between Cytoskeletal (Actin), Plaque (Vinculin), and Membrane (A-CAM) Components of Adherens Junctions: The Effect of Ca^{2+} Depletion

In the next series of experiments the interrelationships between actin, vinculin, and A-CAM were determined. To obtain comprehensive information at the light microscopic level we have double-labeled the same cells for actin and vinculin, actin and A-CAM, or vinculin and A-CAM.

Examination of the double-labeled pairs of unperturbed lens cells revealed the close relationships between actin (Fig. 4, *A* and *C*) and either vinculin (Fig. 4 *B*) or A-CAM (Fig. 4 *D*). In densely plated cultures such as those shown in Fig. 4, focal contacts were relatively sparse and most of the actin filaments were apparently emanating from the vinculin- and A-CAM-containing cell–cell junctions. Vinculin and A-CAM (Fig. 4, *E* and *F*) had nearly identical distributions except for the out-of-focus images of focal contacts (Fig. 4 *E*) detected with antibodies to the former protein only.

Incubation of lens cells with 5 mM EGTA for 10 min has caused remarkable alteration in the organization and distribution of the three junctional proteins. Actin, in EGTA-treated cells, appeared distorted (Fig. 5, *A* and *C*) and its association with the retracting junctional membranes (identified by vinculin [arrows in Fig. 5 *B*] or A-CAM [arrows in Fig. 5 *D*] staining) could not be easily detected. At the margins of cell colonies conspicuous vinculin-containing focal contacts were detectable (Fig. 5 *B*) with F-actin bundles attached to them (Fig. 5 *A*). These structures were relatively resistant to Ca^{2+} withdrawal and remained attached at the cell periphery while the apical aspect of the cells underwent considerable centripetal retraction.

Comparison of vinculin (Fig. 5 *E*) and A-CAM (Fig. 5 *F*) staining in the same EGTA-treated lens cells pointed to an incomplete codistribution at the supraventral focal levels. This has suggested that the association between the two proteins was partially lost after cleavage of the junction, possibly attributable to independent translocations of vinculin, A-CAM, or both.

Recovery of Intercellular Adherens Junctions after Restoration of Extracellular Ca^{2+}: The Effect of Anti-A-CAM Antibody and Its Fab' Fragment

Lens cell cultures were treated with 5 mM EGTA for 10 min as above, until all adherens junctions were apparently opened. The coverslips were then rinsed and transferred into new culture plates supplemented with fresh, Ca^{2+}-containing medium for 5 h more of incubation. Examination of actin, vinculin, and A-CAM in these cells indicated that new intercellular adherens junctions were formed, containing actin (Fig. 6, *A* and *C*), vinculin (Fig. 6, *B* and *E*), and A-CAM (Fig. 6, *D* and *F*). The labeling patterns were somewhat different from those found in untreated cells. This is most clearly demonstrated in the vinculin–A-CAM pair (Fig. 6, *E* and *F*) which reveals A-CAM-containing dots and streaks (see arrows in Fig. 6, *E* and *F*) which are apparently devoid of vinculin. These sites may represent remnants of the "old" junctions, or endocytosed membrane fragments whose subcellular location is not yet known.

Addition of monovalent Fab' fragments of anti-A-CAM to lens cells during the Ca^{2+} recovery phase had a dramatic effect on the recovery process. Phase-contrast microscopic examination indicated that unlike cells undergoing recovery in the presence of DME (Fig. 7 *A*), the cells treated with anti-A-CAM Fab' did not re-form a coherent epithelial sheet (Fig. 7 *B*). Examination of these Fab'-treated lens cells pointed to distinct morphological alterations manifested in considerable rounding up or development of a fibroblastoid appearance in these cells.

Electron microscopic examination of cells after "normal" recovery have revealed a conspicuous, microfilament-associated junction at the subapical region of the cells (Fig. 7 *C*). In the Fab'-treated cells, on the other hand, such junctions

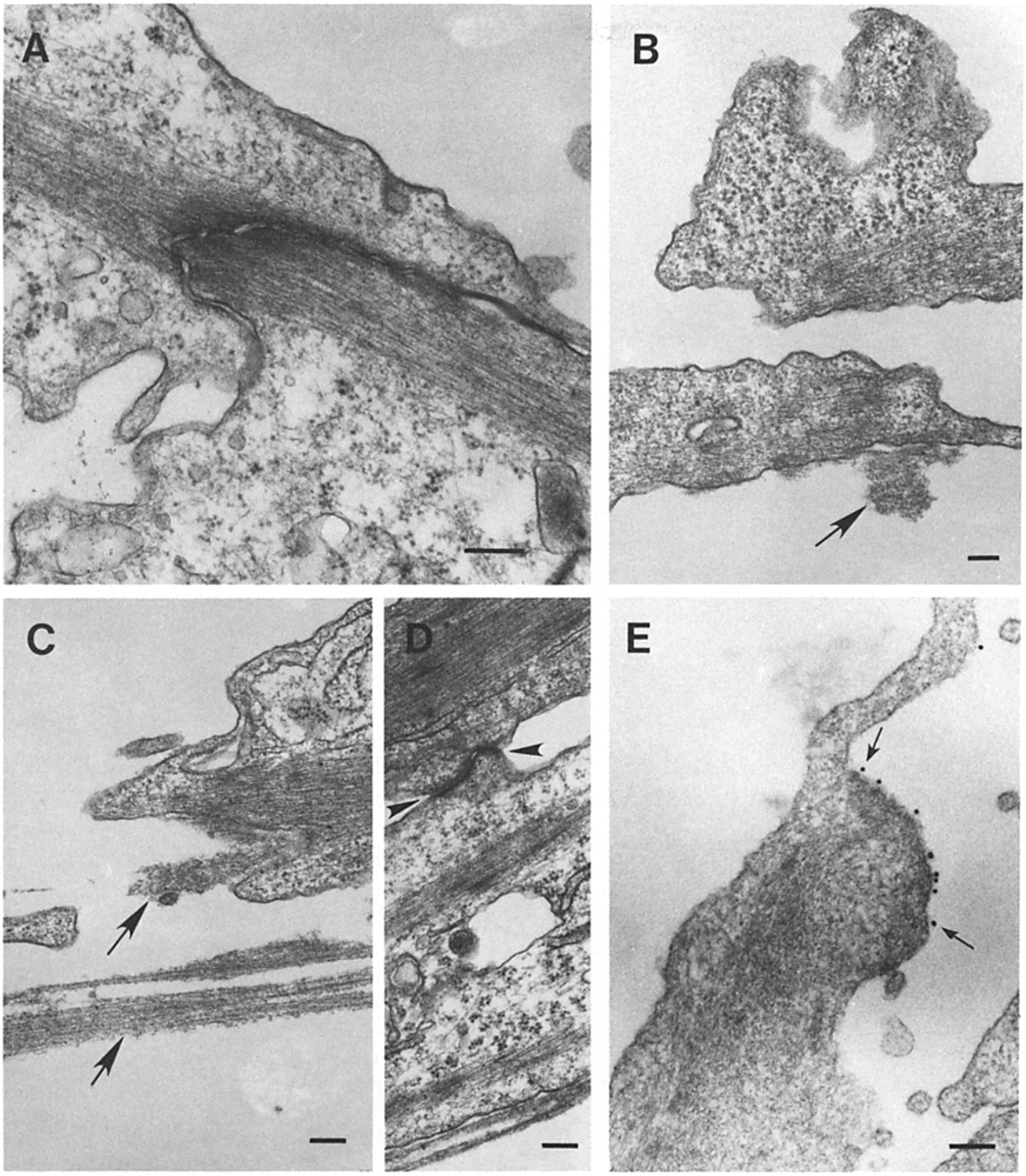

Figure 3. Transmission electron photomicrographs of control (*A*) or EGTA-treated (*B–E*) cultured lens cells. Intact adherens junctions such as the one shown in *A* disappear after addition of EGTA for 10 min (*B–D*) and can rarely be detected even after 90 s of treatment (*E*). Notice that bundles of microfilaments can still be detected at the cytoplasmic faces of the retracting junctional membranes (*B*, *C*, and *E*). Strands of extracellular matrix fibers are often detected throughout the culture as well as in the vicinity of the cleaved junctions (*arrows* in *B* and *C*). It is notable that gap junctions, also present in lens cell cultures, were considerably more resistant than adherens junctions towards Ca^{2+} removal (*arrowheads* in *D*). Localization of A-CAM by immunogold labeling of cultured cells, fixed after short EGTA pulse (*E*). The gold particles appear to be closely associated with the junctional membrane (*arrows*) and an extension of membrane lamella is often detected apically to the labeled region. Bars, 0.2 μm.

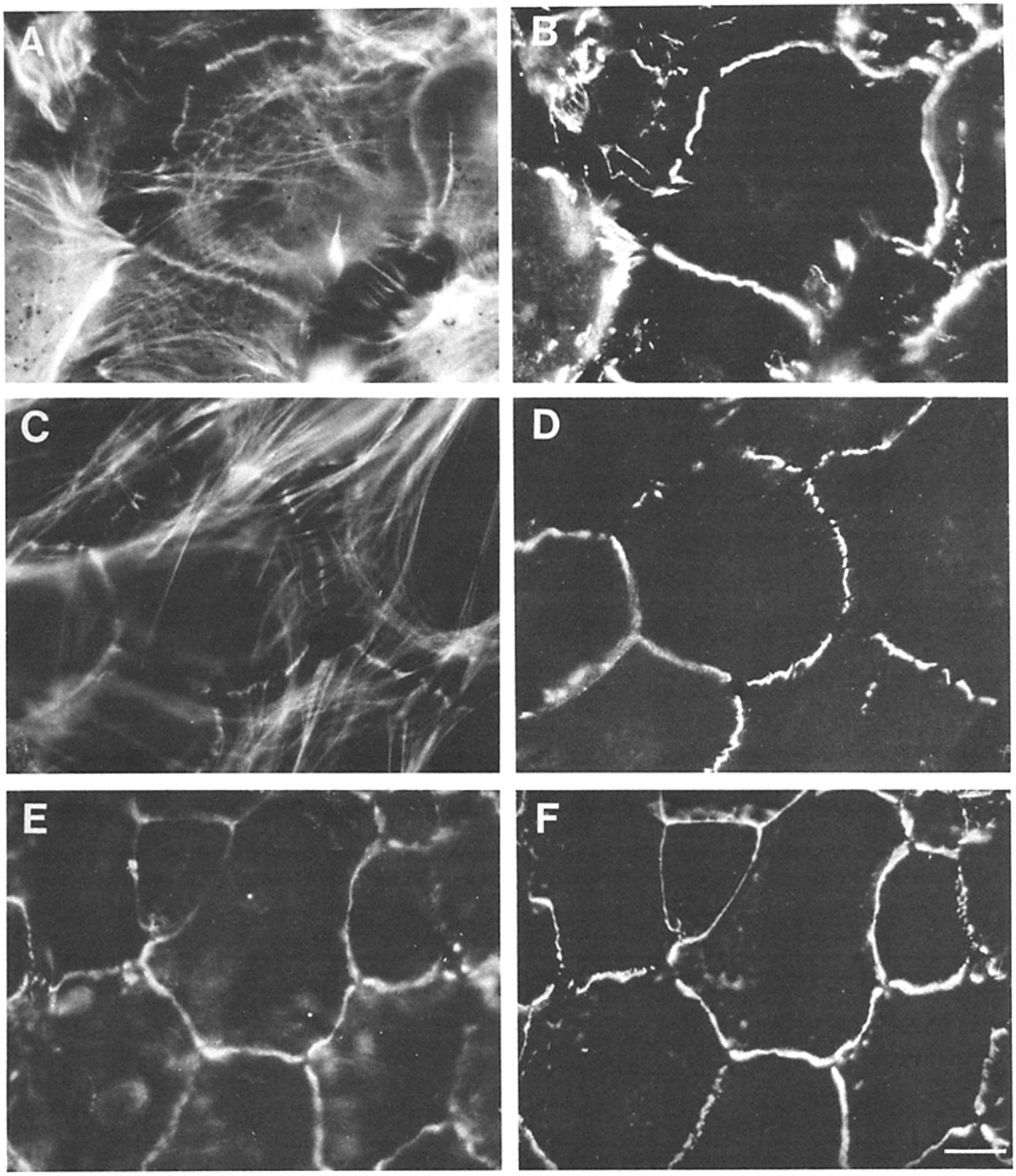

Figure 4. Double immunofluorescent labeling of untreated cultured lens cells for adherens junction-related proteins. (*A* and *B*) Labeling of the same cells for actin (NBD phallacidin) and vinculin (rhodamine, rabbit antibodies), respectively. (*C* and *D*) Labeling of the same cells for actin (as above) and A-CAM (rhodamine, antibody ID-7.2.3), respectively. (*E* and *F*) Labeling for vinculin (rabbit antibodies, fluorescein) and A-CAM (as above), respectively. The focal plane photographed in all these plates was close to the apical surfaces of the cells, yet the ventrally located focal contacts can faintly be seen in the vinculin-labeled cells. Bar, 10 μm.

were not detected. Wherever cell-cell contact was observed no association of microfilament bundles with the membrane was apparent (see arrowheads in Fig. 7 *D*). Moreover, examination of Fab′-treated cells revealed fragments of actin bundles scattered throughout the cytoplasm (arrows in Fig. 7 *E*). These bundles were largely similar to those previously detected in EGTA-treated Madin-Darby bovine kidney cells (38). Examination of these structures at a higher magnification has shown that the deteriorated bundles were closely associated with intermediate filaments (arrowheads in Fig. 7 *F*).

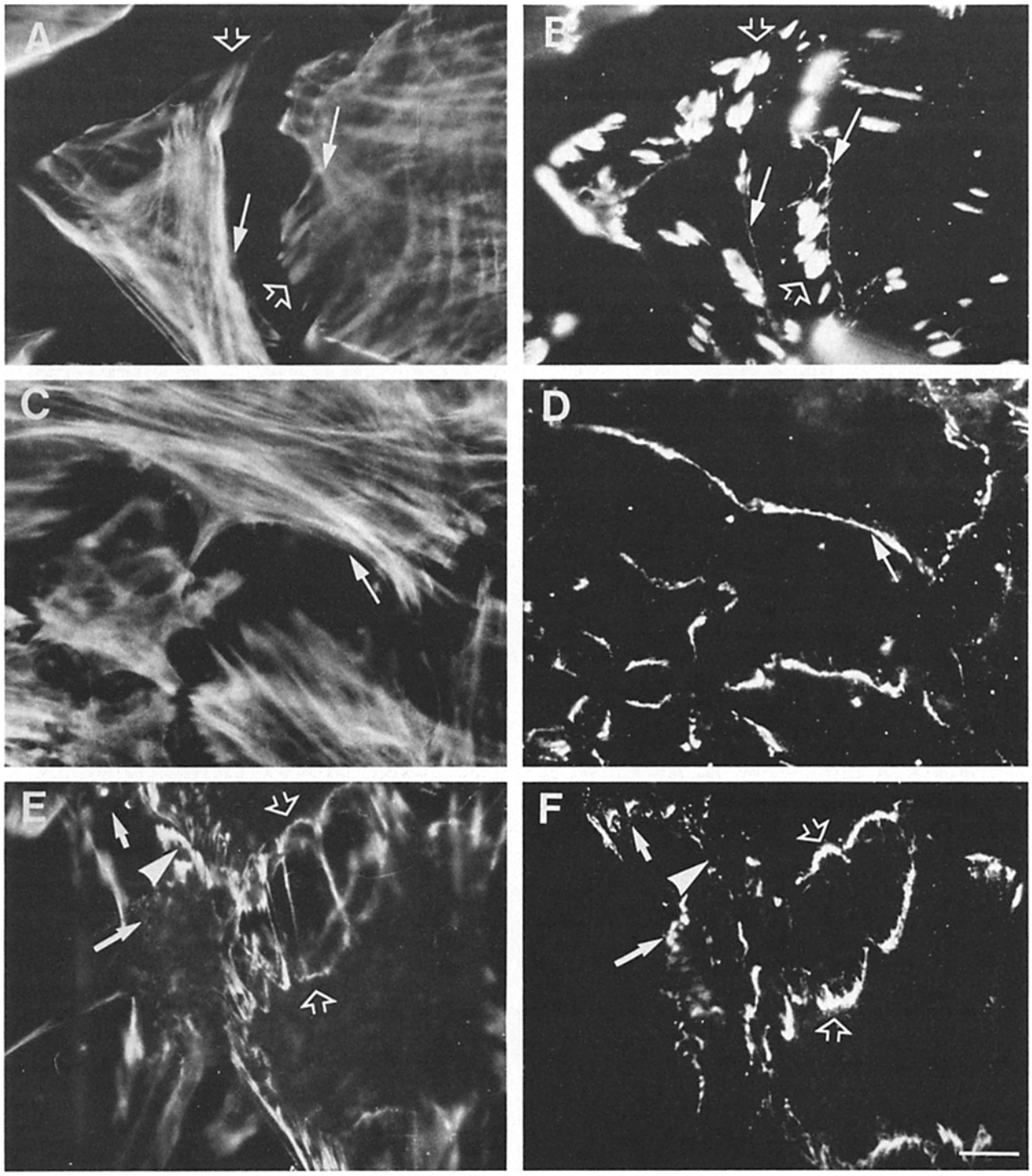

Figure 5. Double immunofluorescent labeling for adherens junction-related proteins in EGTA-treated (5 mM, 10 min) cultured lens cells. The paired labeling patterns shown in *A–F* are as in Fig. 4. Notice the relative resistance of focal contacts to Ca^{2+} removal (matched empty arrows in *A* and *B*) and the pronounced changes that occur in actin organization at the apical focal plane. Actin at the cleaved junction can hardly be detected at the level of resolution of fluorescence microscopy (see matched solid arrows in *A* and *B*, and in *C* and *D*). Comparison of vinculin distributions to that of A-CAM point to extensive areas of coincidence (empty arrows in *E* and *F*) and to regions of exclusive labeling with each protein (sites with vinculin alone marked with arrowheads; sites of A-CAM alone with solid arrows). Bar, 10 μm.

The addition of anti-A-CAM Fab′ to cells during the recovery from EGTA treatment did not only prevent the reestablishment of extensive cell junctions but also caused a dramatic deterioration of the microfilament system as shown in Fig. 8. Actin in cells treated with anti-A-CAM Fab′ (Fig. 8, *A* and *C*) was severely deteriorated, with only a limited number of bundles that were still associated with residual focal contacts (compare actin labeling in Fig. 8 *A* to the corresponding vinculin staining in Fig. 8 *B*). In addition, there were many short cytoplasmic bundles of actin, probably cor-

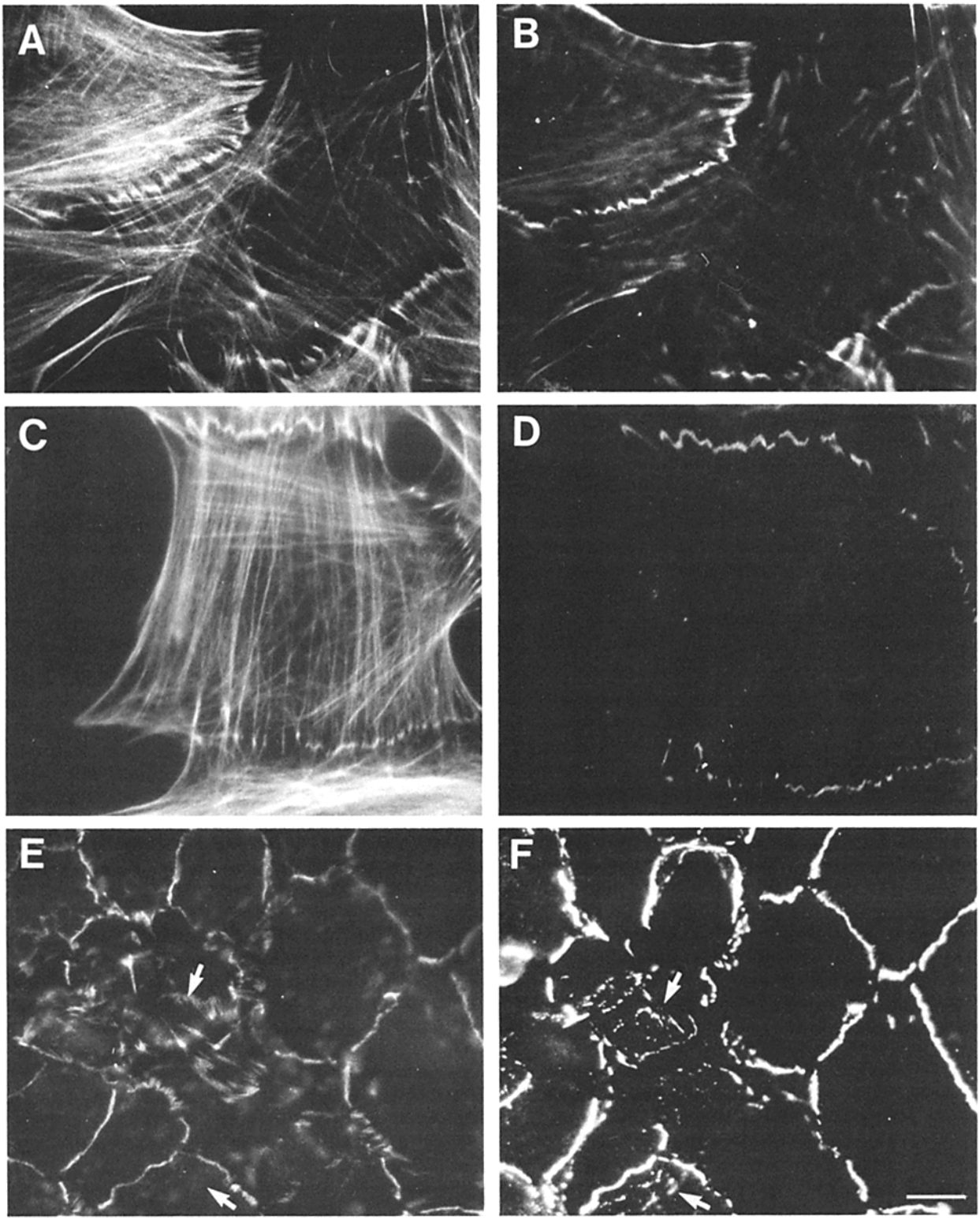

Figure 6. Double immunofluorescent labeling for adherens junction-related proteins of cultured lens cells that have recovered from EGTA treatment for 5 h in normal medium. The paired labeling patterns shown in *A–F* are as described in Fig. 4 above. Notice that intercellular adherens junctions containing actin, vinculin, and A-CAM are re-formed displaying patterns similar (though somewhat disorganized; see text for details) to those found in untreated cells. Arrays of A-CAM-positive dots are occasionally detected in these cells, showing no clear relationship to vinculin or to actin (*arrows* in *E* and *F*). Bar, 10 μm.

responding to the ones shown by electron microscopy in Fig. 7, *E* and *F*. A-CAM exhibited a very low and essentially uniform labeling (Fig. 8, *D* and *F*) showing no relationships to either actin (Fig. 8 *C*) or vinculin (Fig. 8 *E*). Several controls were examined to substantiate the specificity and mode of action of anti-A-CAM Fab′. These included the addition of anti-A-CAM Fab′ to control cells (not treated with EGTA) or the addition of irrelevant Fab′ fragments (anti-acetylcholine

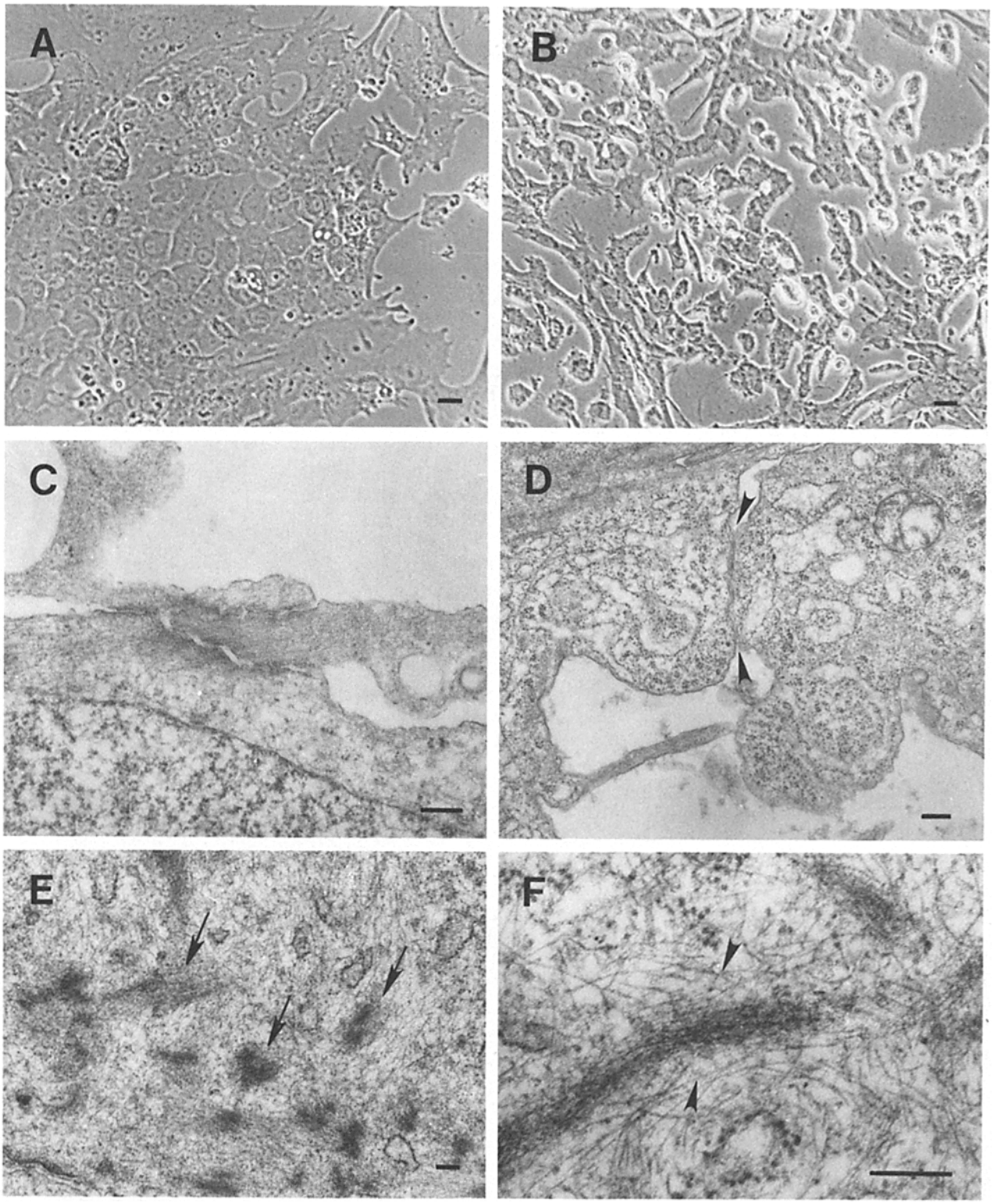

Figure 7. Phase-contrast (*A* and *B*) and transmission electron (*C–F*) micrographs of cultured lens cells undergoing recovery from EGTA treatment in the absence (*A* and *C*) or presence (*B* and *D–F*) of Fab′ fragments of anti-A-CAM. Cultured cells were treated with 5 mM EGTA for 10 min, than washed and further incubated for 5 h with fresh medium with or without the Fab′ fragments (see Materials and Methods). Notice the presence of intact epithelial sheets with well spread cells in the control cultures (*A*). The anti-A-CAM, Fab′-treated cells show rounded or spindle-shaped morphology with considerably fewer cell–cell contacts. The electron micrographs of a control culture (recovered in the presence of DME) show microfilament-bound adherens junctions (*C*) in contrast to the anti-A-CAM Fab′-treated cultures (*D*). The field shown in *D* was selected to demonstrate that even in rare areas of apparent cell–cell contact (between arrowheads) no microfilament bundles can be detected. A common observation in the Fab′-inhibited cells was the abundance within the cytoplasm of disorganized microfilament bundles or bundle fragments (*arrows* in *E*). These fragmented bundles were usually entangled in arrays of intermediate filaments (*arrowheads* in *F*). Bars: (*A* and *B*) 20 μm; (*C–F*) 0.2 μm.

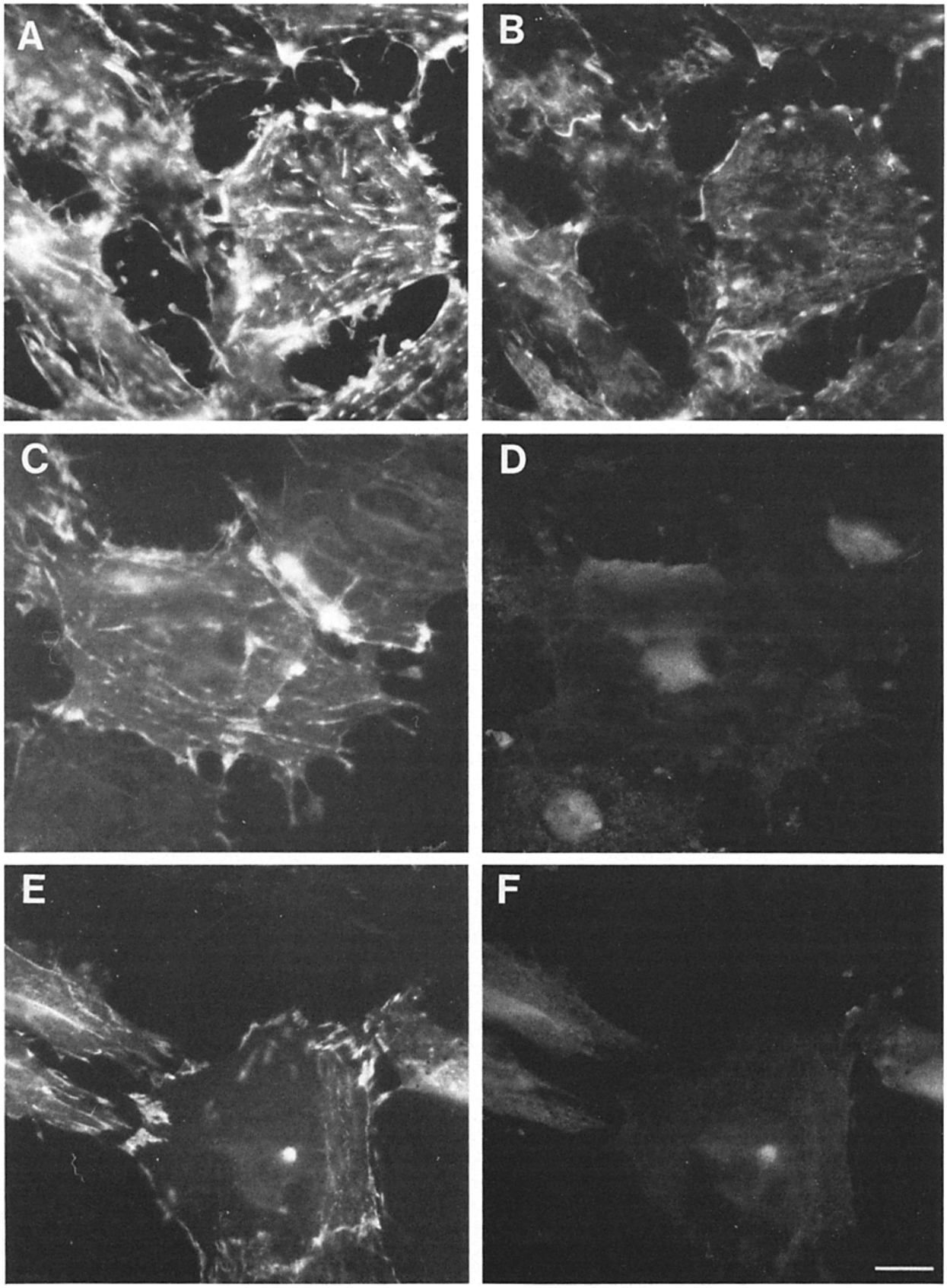

Figure 8. Double immunofluorescent localization of adherens junctions-related proteins in EGTA-treated lens cells after 5 h of incubation with normal medium in the presence of anti-A-CAM Fab' fragments. The paired labeling pattern in *A–F* are as shown in Fig. 4 (*A* and *C*, actin; *B* and *E*, vinculin; *D* and *F*, A-CAM). Notice that the entire microfilament system in the treated cells is severely deteriorated (compare to a control in Fig. 6, *A* and *C*) with only fragmented filament bundles present. Vinculin is similarly disorganized with only residual association with focal contacts. The labeling for A-CAM is weak and uniform throughout the cell periphery. Bar, 10 μm.

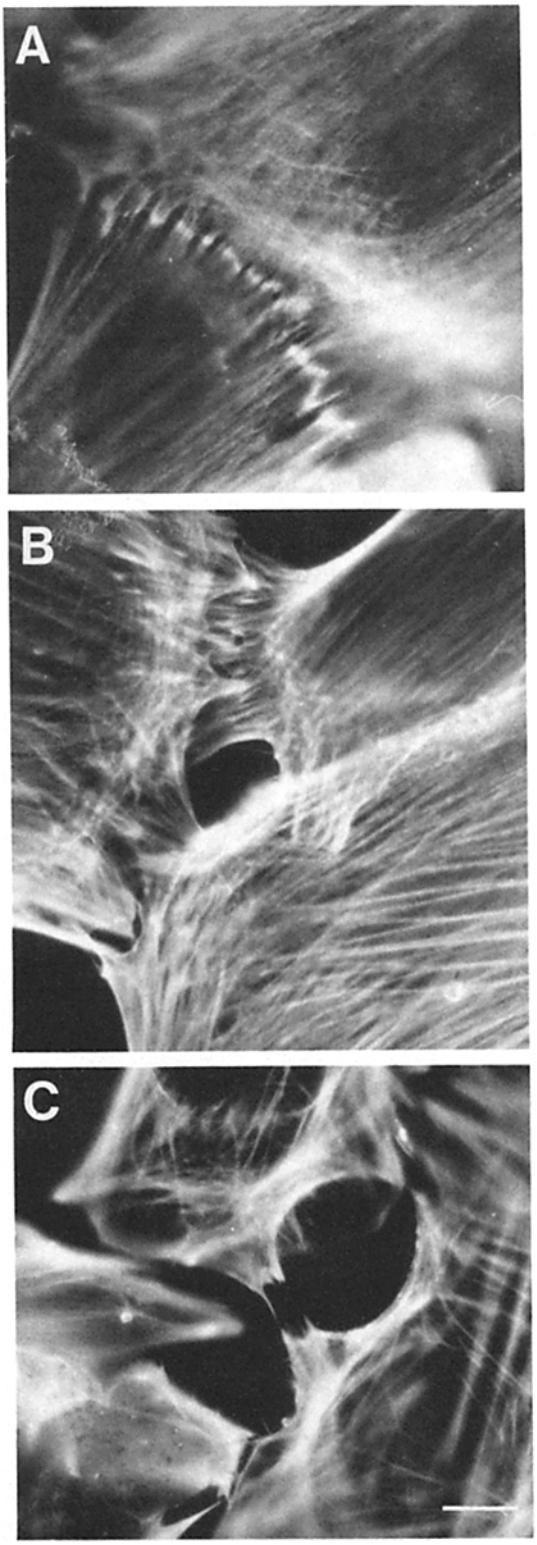

Figure 9. Fluorescent labeling for actin of EGTA-treated lens cells that have recovered their junctions in the presence of intact anti-A-CAM antibodies. (*A*) Cells treated with EGTA (5 mM, 10 min), then incubated for 5 h in the presence of anti-A-CAM. Notice that intercellular adherens junctions were reestablished (unlike the situation in Fab'-treated cells, Fig. 8, *A* and *C*). (*B*) Cells treated with EGTA were recovered as in *A*, then incubated in the presence of 0.5 mM free Ca^{2+} for 5 min. Notice that extensive cell–cell contacts are retained with actin associated with them. (*C*) Cells that have recovered their junctions after EGTA treatment in the absence of A-CAM antibodies (as in Fig. 6, *A* and *C*), then treated with 0.5 mM free Ca^{2+} buffer for 5 min as in *B*. Notice that intercellular contacts in the sample are completely dissociated. Bar, 10 μm.

receptor) to the cells during the Ca^{2+}-dependent recovery of the junction. Both treatments had no apparent effect on junction formation.

An interesting feature of anti-A-CAM effect was noted when EGTA-treated cells were allowed to re-form their junctions in the presence of intact, divalent anti-A-CAM antibodies. Fig. 9 shows that in contrast to Fab', intact antibody had no apparent inhibitory effect on junction re-formation (Fig. 9 *A*). However, in contrast to junctions re-formed in normal medium in the absence of specific antibody, the junctions that were reestablished in its presence became Ca^{2+}-independent. Thus, incubation of these cultures with 0.5 mM free Ca^{2+} solution for 5 min caused only very limited cell retraction and most intercellular adherens junctions remained intact (compare Fig. 9 *B* to *C*). This finding was interpreted as suggesting that the junctions re-formed in the presence of whole IgG contain antibody bridges interconnecting A-CAM molecules of neighboring cells.

Discussion

Previous studies have described the cellular distribution and some of the general molecular properties of the 135-kD protein, denoted here as A-CAM. In our initial study of A-CAM we have established the close spatial interrelationships between this protein and vinculin in cell–cell contact areas (39). Further studies have established the fact that there are two distinct molecular subfamilies of vinculin-rich adherens junctions and that A-CAM is present only in the intercellular junctions (24). In the preceding paper (40) we have extended these studies and determined the fine subcellular localization of A-CAM and some of its molecular characteristics. This information has strongly suggested that A-CAM is a surface glycoprotein of cell–cell adherens junctions in a large variety of mesenchymal, myoid, and epithelial cells (a detailed tissue distribution profile of A-CAM is now in progress).

The present study was directed towards the elucidation of the function of A-CAM in junction biogenesis. This process was postulated, in the past, to play a central role not only in cell adhesion per se but also in the transmembrane nucleation of cytoskeletal organization, mechanical integration throughout tissues, and possibly, the regulation of cell motility, growth, and development (1, 2, 15, 21, 28, 33).

The major evidence for the close involvement of A-CAM in the process of cell–cell interaction is the dramatic effect of anti-A-CAM Fab' on cell–cell adhesion in general and on the formation of adherens junctions, in particular. This type of effect, which has been widely used as a major criterion for the identification of "contact receptors" in the past, appears to be highly specific since irrelevant Fab' had no effect on cell–cell adhesion in the systems tested, and since the intact IgG had no inhibitory effect on junction formation. Moreover, incubation with Fab' did not inhibit cell membrane motility in general and was certainly not toxic to the cells. This was verified by microscopic observations and by the capability of treated cells to recover their adherens junctions after replacement of the Fab' with fresh medium (not shown). Further characterization of cell behavior (mem-

brane motility, locomotory activity, growth, etc.) during the Fab' treatment is presently carried out.

Interesting information was derived from some of the control samples of the Fab' inhibition experiment. This includes the inability of anti-A-CAM Fab' to disrupt intact junctions and the failure of intact (divalent) antibodies to inhibit junction re-formation. The former phenomenon may be attributed to the inaccessibility of the junctional cleft to external proteins and to a low turnover of the junctional proteins (see preceding paper for further discussion [40]). The latter aspect deserves some discussion since it may reveal interesting features of A-CAM-mediated cell adhesion. The apparent differences between the effects of monovalent and divalent anti-A-CAM antibodies are most likely attributable to the valency and not, for example, to altered or reduced binding capacity of the latter. Conceptually the binding of the whole IgG to the exposed surface of the cleaved junction should be at least as avid as that of the Fab' fragment. Moreover, previous experiments in which EGTA-treated, nonpermeabilized cells were labeled with intact antibodies showed an extensive labeling of the two hemi-junctions (39). A likely explanation of the inability of intact antibody to inhibit junction assembly is that it actually bridges between A-CAM molecules of neighboring cells and essentially "replaces" the normal receptor-mediated interactions. This interpretation was strongly corroborated by the fact that adherens junctions that were reestablished in the presence of anti-A-CAM became Ca^{2+}-independent (Fig. 9). It is noteworthy that the "anti-A-CAM-mediated junction" can not only mechanically hold the cells together but also effectively organize locally the cytoskeleton (actin bundles) through a vinculin-rich plaque in a mode that is indistinguishable by light and electron microscopies from that of normal junctions. In our previous studies we have proposed that the interaction of microfilament bundles with the junctional membrane is locally induced by a primary, receptor-mediated interaction with the external surface (for discussion of that aspect see references 20 and 21). If that concept is accepted one may further postulate that the signals for microfilament (or vinculin) assembly are transmitted across the membrane via changes in aggregation or immobilization of the "adherens junction-specific receptor(s)". Thus, A-CAM-mediated cell-cell binding may lead to actin reorganization, whereas interactions in the same cells mediated by other membrane receptors, such as gap junction proteins (in lens cells) or desmosomal components (in cardiac or kidney cells), do not.

In principle the specific intercellular interaction in which A-CAM is involved could be of a homophilic or heterophilic nature (for discussion of the two types of adhesion, see references 13 and 14). The presently available information is too preliminary to directly determine which of the two predominates. Yet, the results discussed above suggest that the antibody-mediated homophilic interactions that are probably induced by anti-A-CAM mimic the organization of unperturbed junction leading to both cell adhesion and to the induction of transmembrane reassembly of the cytoskeleton. Additional studies will be necessary to establish whether the native interactions of A-CAM are also of the homophilic type.

We would like to briefly discuss here another aspect, namely the cellular, molecular, and functional relationships of A-CAM to other cell adhesion molecules that have been studied in recent years. Most adhesion-related molecules so far studied were initially identified and characterized using a functional assay such as the capability of specific antibodies to disrupt cell contacts or to prevent their formation. This approach has revealed families of cell adhesion molecules that could further be grouped according to their molecular weights, tissue specificity, Ca^{2+}-dependence, and programmed expression during embryogenesis. Little attention has been devoted so far to the fine subcellular locations of the various CAMs in cells and tissues. Only recently it has been claimed that uvomorulin (29) and a related protein detected by a monoclonal antibody, anti-Arc-1 (5), are present in the zonulae adherentia of intestinal epithelium and Madin-Darby canine kidney cells, respectively (6). It should, however, be pointed out that other studies carried out recently on the Madin-Darby canine kidney junction suggest that uvomorulin is not strictly confined to the junctional area but rather displays a broad distribution over the entire basolateral membranes (26). The molecular and cellular bases for this controversy are not clear yet.

Our primary antibody selection strategy (see reference 39) was quite different, looking for junction-associated rather than contact-disrupting antibody activity. Retrospectively, a functional screening (as mentioned above) for the hybridoma supernatants would not have revealed our anti-A-CAM antibodies since they do not disrupt existing cell adhesions.

The spectrum of CAMs has become too wide to allow for their detailed comparison to A-CAM. We would like therefore to briefly discuss here only those adhesion molecules that share some properties with A-CAM and especially those that participate in a Ca^{2+}-dependent cell-cell adhesion. The most extensively studied CAM with such characteristics is uvomorulin (29, 37), also known as L-CAM (9, 16), cell CAM 120/80 (10), E-cadherin (32, 41, 42), and Arc-1 (5). These contact receptors were reported to have molecular weights somewhat lower than that of A-CAM (in the range of 120–124 kD) and have an ~80-kD stable proteolytic fragment (5, 9, 10, 29) that is reminiscent of the 78-kD, cell-bound tryptic fragment of A-CAM (see preceding paper [40], Fig. 4). In addition they show strict Ca^{2+}-dependence and are apparently Con A–binding glycoproteins that do not bind to wheat germ agglutinin (9, 29).

There are, however, some distinct differences between A-CAM and the uvomorulin family that should be pointed out here. The most prominent one is the distinctly different tissue-specific expression of the two receptors; the original cell type in which uvomorulin has been identified was mouse embryonal carcinoma cells (29). In the adult chicken, L-CAM was detected in a variety of epithelia, both simple and stratified (for details see Edelman [14]), and a most prominent source for it was the liver. A-CAM, on the other hand, is apparently absent from polarized epithelia of intestine, liver, and kidney tubules and prominently expressed in cardiac muscle, eye lens, brain, and cultured kidney cells (24, 39). Although the immunohistochemical survey of A-CAM is still incomplete, it appears that the two CAMs are differentially expressed in distinct cell types. The only apparent overlap is in kidney, though this may be due to the presence of A-CAM and uvomorulin in distinct cell populations or at different developmental stages. Another difference in the properties of uvomorulin-mediated and A-CAM-mediated junctions is manifested by the effect of antibodies on junction integrity.

In contrast to anti-A-CAM, the junctions mediated by the uvomorulin-related molecules were disrupted by the intact antibody used (5, 10, 32, 37) and in most cases the preceding EGTA-induced cleavage of the junction was not necessary (5, 10, 29, 32). It is still not clear whether these differences are attributable to the topology of the different junctions, or to the accessibility and functional involvement of the particular antigenic epitopes recognized by the different antibodies used in these studies. Additional explanation is that there are differences in the turnover rate (residence period) of A-CAM and L-CAM within the junctional domain. In conclusion, we propose that the uvomorulin-related cell adhesion molecules and A-CAM are molecularly distinct contact receptors that are differentially expressed in cells and tissues yet may participate in the construction of structurally related junctions. Further comparative characterization of the two is presently in progress.

Additional cell adhesion–related proteins, which should be compared here to A-CAM, are the recently described N-cadherin (27) and the 130-kD adhesion molecule of embryonic neural retina (25). Concerning the former, at least some of its properties are similar to those of A-CAM. These include an apparent Ca^{2+}-dependence, at least partially overlapping tissue-expression profile, and a similar molecular weight. Immunoblot reactions using our antibodies and anti-N-cadherin obtained from Dr. M. Takeichi (Kyoto University, Japan) revealed similar migration of the positively reacting bands. A notable difference was the exclusive binding of our antibodies to the major proteolytic fragments, suggesting that if the two antibodies bind to the same molecule, they recognize different epitopes (or regions) on it. Some uncertainties still exist as to details in their respective tissue distributions (part of which may be related to differences in species studied) and fine subcellular localization. These include the reported presence of N-cadherin in skeletal muscle, the presence of A-CAM in kidney cells, and the absence of information on the detailed microscopic distribution of N-cadherin. Similar considerations may also be applied for the calcium-dependent adhesion protein from chick neural retina described by Grunwald et al. (25). Attempts are presently made to further characterize the cellular and molecular properties, broad tissue distribution profile, and developmentally regulated expression of A-CAM. Hopefully these studies will shed further light on the process of cell adhesion in general and the biogenesis of intercellular adherens junctions in particular.

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