

Molecular Heterogeneity of Adherens Junctions

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ABSTRACT We describe here the subcellular distributions of three junctional proteins in different adherens-type contacts. The proteins examined include vinculin, talin, and a recently described 135-kD protein (Volk, T., and B. Geiger, 1984, *EMBO (Eur. Mol. Biol. Organ.) J.*, 10:2249–2260). Immunofluorescent localization of the three proteins indicated that while vinculin was ubiquitously present in all adherens junctions, the other two showed selective and mutually exclusive association with either cell–substrate or cell–cell adhesions. Talin was abundant in focal contacts and in dense plaques of smooth muscle, but was essentially absent from intercellular junctions such as intercalated disks or adherens junctions of lens fibers. The 135-kD protein, on the other hand, was present in the latter two loci and was apparently absent from membrane-bound plaques of gizzard or from focal contacts. Radioimmunoassay of tissue extracts and immunolabeling of cultured chick lens cells indicated that the selective presence of talin and of the 135-kD protein in different cell contacts is spatially regulated within individual cells.

On the basis of these findings it was concluded that adherens junctions are molecularly heterogeneous and consist of at least two major subgroups. Contacts with noncellular substrates contain talin and vinculin but not the 135-kD protein, whereas their intercellular counterparts contain the latter two proteins and are devoid of talin. The significance of these results and their possible relationships to contact-induced regulation of cell behavior are discussed.

Adherens junctions consist of a family of stable cell contacts in which actin is characteristically associated with the endofacial surfaces of the plasma membrane (16, 20, 23, 40). Typical examples of adherens junctions are the *zonula* and *fascia adherentes* of polarized epithelia and cardiac myocytes (32, 42), small adhesions of fibroblasts (29), focal contacts in cell cultures (3, 18), dense plaque of smooth muscle (19), etc. The major justification for the reference to all these morphologically diverse structures as a closely related group of cell contacts was the apparent molecular homology between them. Studies in several laboratories indicated that all adherens junctions contain vinculin at their cytoplasmic aspects, and it was thus postulated that vinculin is involved in the linkage of actin to the membrane in these sites. The presence of a ubiquitous “plaque” component in all adherens junctions and their apparent association with actin filaments have raised the possibility that, in spite of considerable morphological variability, the molecular homology between all adherens junctions may be quite extensive. To study this aspect directly we have tried to identify additional components of adherens junctions and study their spatial distributions in cells and tissues.

Recently two relevant proteins were described which are specifically bound to adherens junctions, including talin (9, 34) and a 135-kD protein (45). The former is a 215-kD protein isolated initially from chicken smooth muscle. Immunofluorescent labeling of cultured chick cells with talin-specific antibodies yielded patchy patterns along the ventral cell surfaces which were nearly identical to those of vinculin. The requirement for cell permeabilization as well as the relative resistance of talin towards treatment with actin-severing proteins such as fragmin (22) suggested that talin, like vinculin, is a component of the junctional plaque.

The 135-kD protein was identified with a monoclonal antibody (mAb)¹ raised against integral membrane proteins of chick cardiac intercalated disks (45). Immunofluorescent localization indicated that these mAb's, designated ID 7-2.3, react specifically with the intercalated disks of chick cardiac muscle as well as with intercellular contacts of cultured myocytes or lens cells.

In the present study, we compared the distributions of talin,

¹ *Abbreviations used in this paper:* mAb, monoclonal antibody.

vinculin, and the 135-kD protein in various cells and tissues. We show here that while vinculin is ubiquitously present in all adherens junctions, the 135-kD protein is associated only with intercellular contacts and talin with attachments to non-cellular matrices exclusively. This molecular heterogeneity of adherens junctions appears to be spatially regulated since both types of junctions may co-exist within the same individual cells.

MATERIALS AND METHODS

Immunochemical Reagents: Anti-vinculin was prepared in rabbits (polyclonal) or in mice (monoclonal), and affinity purified on Sepharose-bound chicken gizzard vinculin (18). Rabbit antibodies to talin were kindly provided by K. Burridge from the University of North Carolina at Chapel Hill. In some experiments we used mAb's raised by us against chicken talin. The specificity of these mAb's was determined by immunofluorescent labeling and immunoblotting analyses (unpublished data). As secondary antibody reagents we used goat anti-rabbit Ig or 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 (4, 8). Fluorescent labeling of actin was performed using rhodamine-phalloidine kindly supplied by Dr. H. Faulstich, from the Max-Planck Institute, Heidelberg, FRG (47).

Immunohistochemical Labeling: Thin frozen sections (0.5–1 μm) of chicken gizzard, heart, and lens were prepared according to Tokuyasu (41 [see also reference 27]) in the Sorvall MT2B ultramicrotome with a cryoattachment. The sections were retrieved with a platinum loop in 2.3 M sucrose

droplets and indirectly immunofluorescently labeled as described earlier (17). Lens cell cultures were prepared from 6–8-d-old chick embryos and subcultured on 18-mm coverslips. Most of the cultured cells exhibited an epithelioid morphology and formed dense sheets. For immunofluorescent labeling, cells were permeabilized by a 2-min exposure to 0.5% Triton X-100 in 50 mM morpholinoethane sulfonate buffer, 3 mM EGTA, 5 mM MgCl₂, pH 6.0, and fixed for 30 min with 3% paraformaldehyde. Double-immunofluorescent labeling was carried out, largely as described (18) using, in conjunction, mouse and rabbit antibodies. It was routinely verified that the secondary goat antibodies were exclusively reactive with their respective antigens.

Immunoelectron Microscopy: Chicken gizzard was dissected into square, 1-mm blocks in 3% paraformaldehyde containing 0.1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). After a 1-h fixation, the tissue blocks were rinsed and incubated for at least 1 h in 0.9 M sucrose. Ultrathin frozen sections of 600 Å thickness were then cut in the Sorvall ultracryomicrotome as described above. The sections were recovered on 300 mesh grids and immunolabeled indirectly with talin antibodies and gold-conjugated (10 nm) goat anti-rabbit IgG (Janssen Pharmaceuticals, Beerse, Belgium).

Quantitative Immunochemical Determination of Vinculin 135-kD Protein and Talin in Chicken Tissues: Chicken heart, lens, and gizzard were homogenized in 10, 15, and 30 volumes, respectively, of RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% deoxycholate, 1% Triton X-100, pH 7.2) and the non-extractable residue removed by a 30-min centrifugation in an Eppendorff microcentrifuge. The supernatant was applied in threefold dilutions to V-shaped multiwell plates (Dynatech Laboratories, Inc., Alexandria, VA) for 30 min at 4°C. After extensive rinsing in PBS containing 1% bovine serum albumin, monoclonal mouse antibodies (hybridoma supernatant used at various dilution between 1:20 and 1:2,000) were added in 50 μl for 1 h to the wells. The wells were then rinsed, incubated with

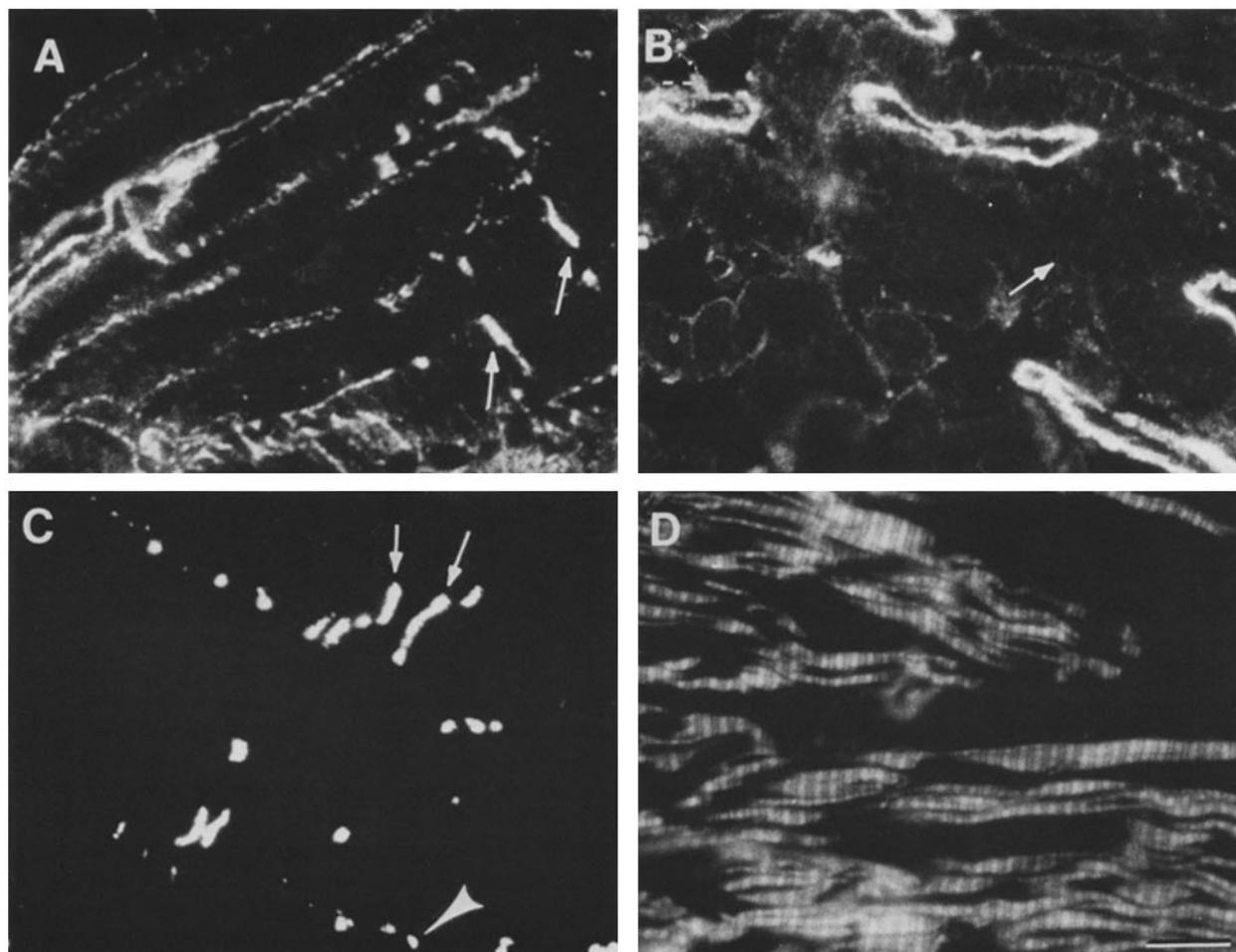


FIGURE 1 Immunofluorescent localization of vinculin (A), talin (B), the 135-kD protein (C), and actin (D) in thin frozen sections of chicken cardiac muscle. The arrows point to intercalated disk-containing areas, identified by phase-contrast microscopy. The arrowhead in C points to contact sites along the lateral cell membranes. Notice the extensive labeling of intercalated disks for vinculin and for the 135-kD protein and the apparent absence of labeling of these sites with talin antibodies. Bar, 10 μm.

¹²⁵I-labeled goat anti-mouse Ig (70,000 cpm/well), rinsed again, and counted in a gamma counter (Kontron AG Analytical Export, Zurich, Switzerland).

RESULTS

Localization of Microfilament-associated Proteins: Actin, Vinculin, Talin, and the 135-kD Protein in Cardiac Muscle, Smooth Muscle, and Eye Lens

We have localized the various junctional proteins in three different chick tissues: heart, gizzard, and lens. Localization of vinculin in heart tissue using either affinity-purified antibodies or a mAb revealed two major patterns of labeling: most prominent staining was found along the intercalated disks (Fig. 1A, arrows). In addition, periodic lateral dots were detected, corresponding to "costameric" organization as previously described (13, 37, 38). Talin antibodies were mostly negative in the cardiac muscle tissue with occasional staining of the lateral dots, but gave an extensive labeling of the

vascular smooth muscle, as shown in Fig. 1B. Staining with mAb ID 7-2.3 (anti-135-kD protein) showed a restricted labeling confined mostly to the intercalated disks (Fig. 1C). Comparison of the labeling pattern to the phase-contrast image of the section was shown in reference 45. Actin, visualized with rhodamine-phalloidin, exhibited a typical striated pattern corresponding to the sarcomeric periodicity in the myocytes with relatively little labeling throughout the connective tissue (Fig. 1D).

Different patterns of immunolabeling for the different proteins was obtained with chick gizzard smooth muscle. Both vinculin and talin (Fig. 2, A and B) exhibited indistinguishable dotted labeling along the cell periphery. Staining with anti-135-kD protein was essentially negative (Fig. 2C), whereas extensive labeling of the smooth muscle was obtained with rhodamine-phalloidin. The labeling of the interspacing connective tissue was relatively low (Fig. 2D). To directly identify the site of talin localization at the periphery of the smooth

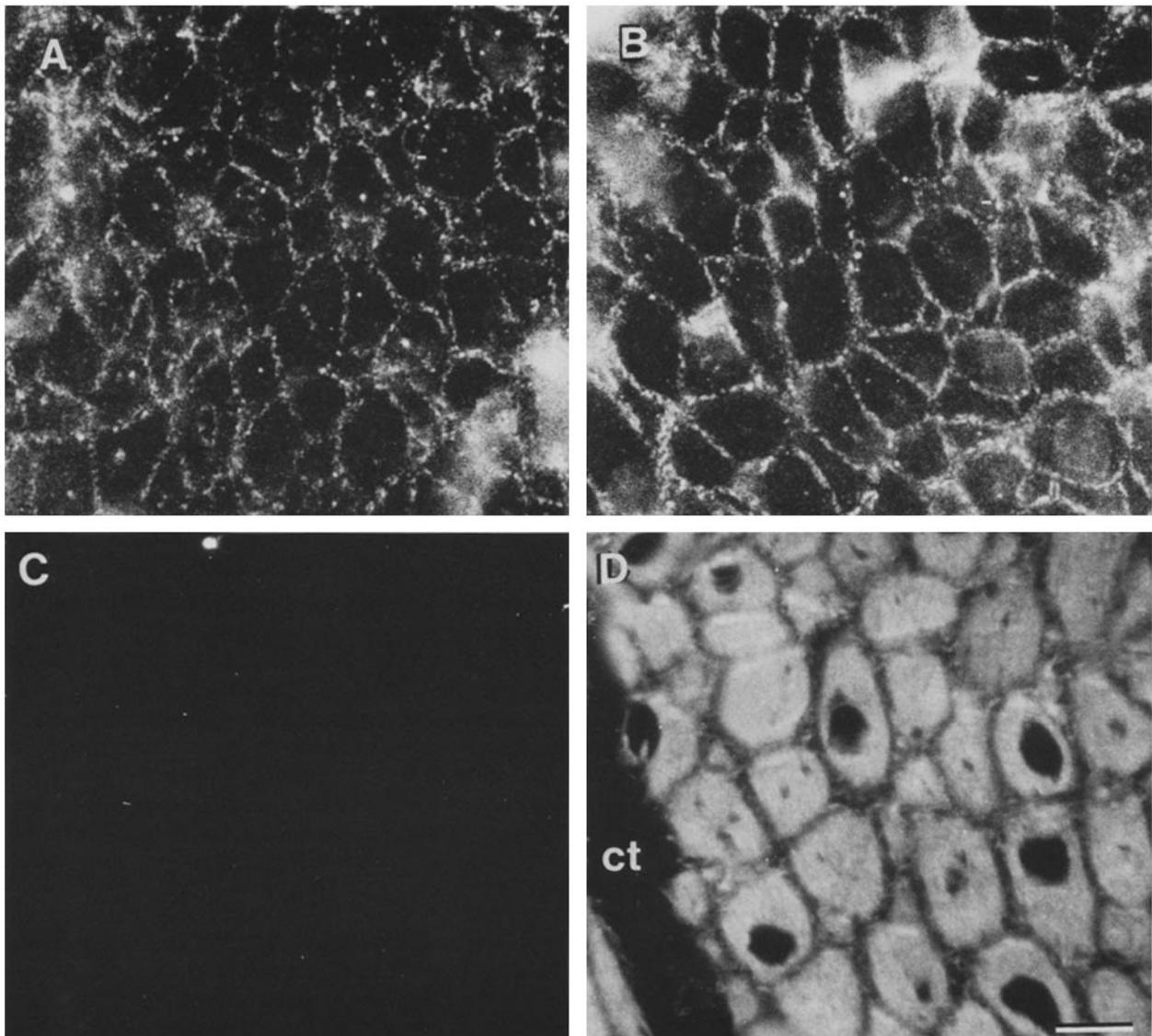


FIGURE 2 Immunofluorescent localization of vinculin (A), talin (B), the 135-kD protein (C), and actin (D) in thin frozen sections of chicken gizzard smooth muscle. Notice the punctate labeling for vinculin and talin along the cell membranes and the apparent absence of labeling for the 135-kD protein. Actin was abundant throughout the cytoplasm of muscle cells with very little labeling of the connective tissue (ct). Bar, 10 μ m.

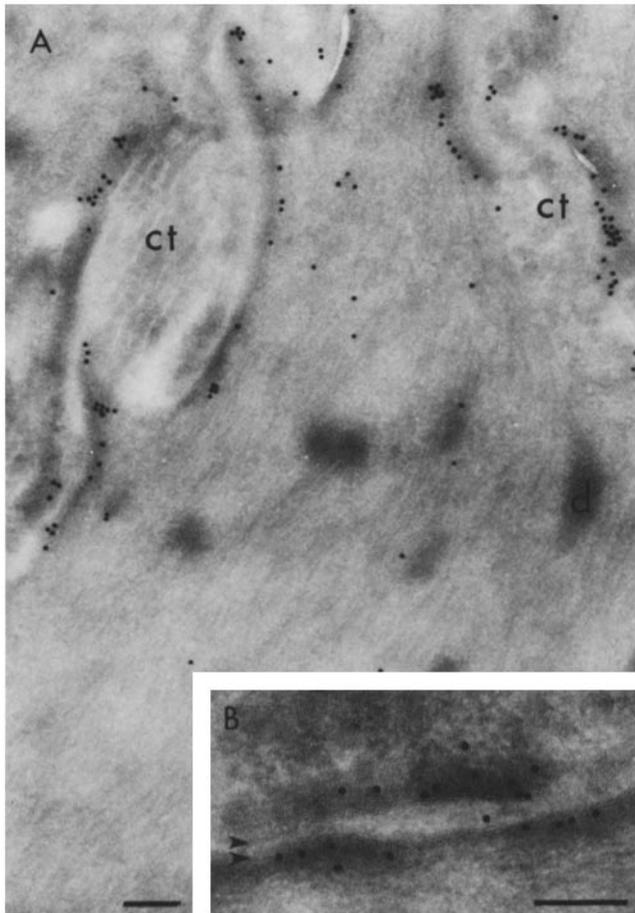


FIGURE 3 (A and B) Immunogold labeling of ultrathin frozen sections of chicken gizzard smooth muscle for talin shown at two levels of magnification. Sections were labeled with rabbit antibodies followed by secondary goat anti-rabbit Ig coupled to 20-nm gold particles. The labeling was restricted predominantly to the membrane-bound dense plaques and was absent from cytoplasmic dense bodies. (The cell membranes in B are indicated by the arrowheads). In the intercellular space collagen fibers (ct) are commonly detected. It has been previously shown by immunoelectron microscopy (19) that vinculin is similarly associated with the dense plaques of smooth muscle. Bar, 0.2 μ m.

muscle cells, we have prepared ultrathin frozen sections of gizzard tissue and immunolabeled them indirectly with anti-talin and gold-conjugated secondary antibodies. As demonstrated in Fig. 3, specific labeling was detected on the membrane-bound dense plaque, a structure previously shown to contain vinculin (19).

In chicken lens, an intense labeling along the cell membrane was obtained with vinculin-specific antibodies (Fig. 4A), with anti-135-kD protein (Fig. 4C), and with rhodamine-phalloidin (Fig. 4D). In contrast to smooth muscle, no specific labeling was obtained for talin in the lens tissue (Fig. 4B).

The results described above (in particular, the apparent absence of organized 135-kD protein from gizzard and talin from lens) prompted us to examine whether the proteins were actually absent or whether their organization was modified. Since the 135-kD protein has not yet been isolated in pure form, we have used an indirect approach to quantitate the relative amounts of the three proteins in the different tissues. Serial threefold dilutions of tissue extracts were immobilized

on multi-well microtiter plates and an indirect solid-phase binding assay was performed. At relatively high extract concentrations, a plateau in the level of binding was obtained while at higher dilutions the level of antibody binding declined linearly. Since our attempt was to compare the relative quantities of each of the three antigens in the different tissues, we have applied all extracts at concentrations yielding 50% saturation in binding with anti-vinculin at a fixed dilution and compared the half-saturation dilutions for each of the other antigens. This analysis provided an estimate for the relative levels of the various proteins in the extracts. As shown in Fig. 5, cardiac muscle showed comparable binding profiles for vinculin, the 135-kD protein, and talin. In lens, on the other hand, antibodies to vinculin and to the 135-kD protein showed similar binding while considerably lower levels of binding were obtained with anti-talin. The picture was almost reversed in chicken gizzard where high levels of talin were detected as compared with considerably smaller concentrations of the 135-kD protein. It should be emphasized that essentially the same picture was obtained at different antibody concentrations. It was therefore concluded that the relative amounts of the three proteins in the tissues examined are compatible with the levels of labeling obtained by immunohistochemistry, yet the three proteins were present in all three tissues examined.

Spatial Segregation of Adherens Junction Proteins in Cultured Cells

To further study the selective segregation of the different junction-associated proteins at a higher level of resolution, we have double-immunolabeled cultured chicken lens cells for the 135-kD protein and actin (Fig. 6, A and B). Comparison of the fluorescent patterns indicated that the distribution of the former protein was restricted to cell-cell contact areas from which stress fibers apparently emanated. Double-labeling for the 135-kD protein and vinculin (Fig. 6, C and D) demonstrated that the former was present in intercellular contacts only and was apparently absent from vinculin-rich cell-substrate focal contacts.

Distinctly different were the patterns of organization of talin within cultured lens cells. In individual adherent cells (Fig. 7, A and B) the distribution patterns of talin and vinculin were essentially identical, both presenting extensive association with cell substrate as previously described (9, 10). However, in denser areas along the culture (Fig. 7, C-F), vinculin was apparently present also at the intercellular junctions of the cells (Fig. 7, D and F). In some areas the junction-associated vinculin was elongated while in others it consisted of series of peripheral dots. At later stages, when the cultures reached complete confluency, the junctional belt was nearly continuous along the entire cell periphery as shown in Fig. 6 and Fig. 8. Direct examination of talin distribution, in the same cells, indicated that it was associated with focal contacts exclusively, and was not present at the vinculin-containing intercellular junctions (compare Fig. 7, C and E, with D and F). Moreover, double-fluorescent labeling of cultured chick lens cells for actin and talin indicated that the talin-rich plaques were associated only with that population of actin bundles, which is attached to the ventral cell membrane and was not present at the sites of actin anchorage in the intercellular contacts (not shown). To directly determine the spatial relationships between talin and the 135-kD protein we have

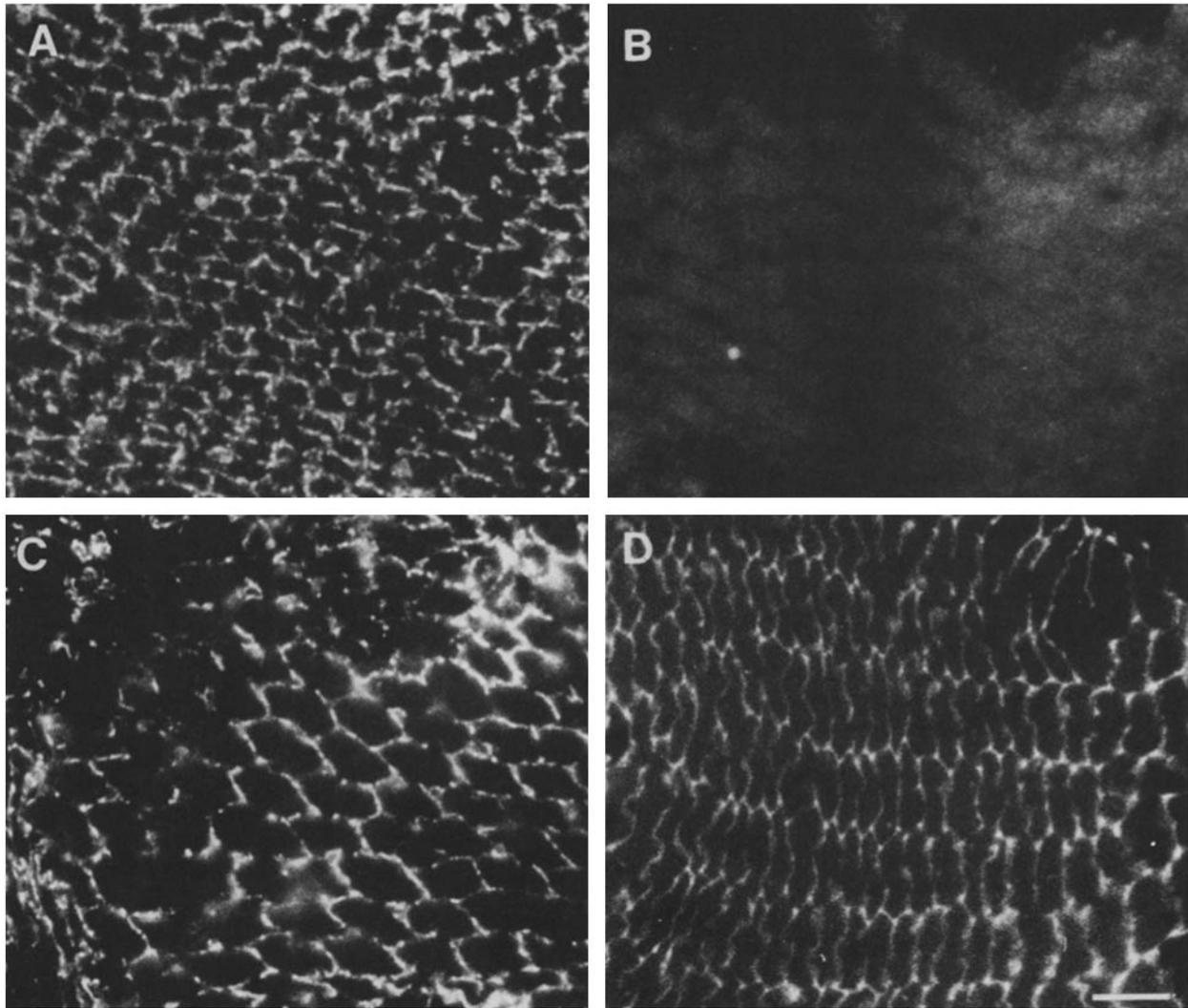


FIGURE 4 Immunofluorescent localization of vinculin (A), talin (B), the 135-kD protein (C), and actin (D) in thin frozen sections of chicken eye lens. The honeycomb pattern of the lens fibers is readily apparent in the cross or slightly tangential sections. Notice that vinculin, the 135-kD protein, and actin are all found predominantly at the cell periphery, the former two displaying a spotty pattern. No detectable labeling is obtained with talin-specific antibodies. Bar, 10 μm .

double-immunolabeled cultured chicken lens cells for the two proteins. Examination of the immunofluorescent patterns pointed to the mutually exclusive associations of the 135-kD protein with intercellular junctions (Fig. 8, *B* and *D*) and of talin with cell-substrate contacts (Fig. 8, *A* and *C*).

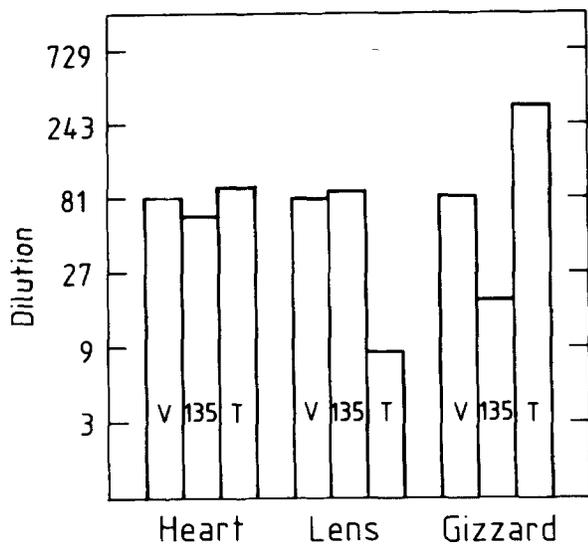
DISCUSSION

Extensive efforts have been directed in recent years towards the molecular characterization of cell junctions. For over two decades, the various defined cellular junctions and adhesions were characterized morphologically and part of their involvement in physiological processes was illuminated (see reference 40). Lately many attempts have been made to isolate intact junctions and to study their composition and biogenesis. Recent advances are largely attributable to the progress made during the last decade in the characterization of the cytoskeleton, the development of methods for subcellular fractionation and the preparation of antibodies reactive with junctional components. In such studies the molecular properties of gap junctions were partially characterized (14, 24, 25, 31) as well

as those of desmosomes (12, 15, 26, 35, 39) and adherens junctions (20, 23).

The adherens junctions represent a widely spread family of cell contacts in which actin is locally associated with the endofacial surfaces of the membrane. The findings that vinculin is ubiquitously associated with all adherens junctions examined, regardless of whether they were formed with neighboring cells or with noncellular surfaces and irrespective of their fine ultrastructure, were the basis for the suggestion that all adherens junctions share considerable molecular homology (20, 22).

To examine this hypothesis, we have previously fractionated various adherens junctions into several molecular subdomains which could be experimentally modulated (5, 23). We have shown that selective severing of membrane-bound actin by fragmin leads to destruction of microfilaments and the concomitant removal of many actin-associated proteins (α -actinin, tropomyosin, myosin, filamin) leaving the vinculin-rich plaque of focal contacts apparently unaffected. Alternatively, dissociation of cell-cell contacts of cultured epithelial cells by the removal of extracellular Ca^{2+} induced a



coordinated detachment of both vinculin and actin from the junctional membrane (20, and Volberg, T., B. Geiger, J. Kartenbeck, and W. W. Franke, manuscript in preparation).

As pointed out above, many attempts have been made to identify and isolate additional adherens junction proteins and in particular integral constituents of the junctional membrane. Two such components have recently been identified. Burridge and Connell have isolated talin, a 215-kD cytoplasmic protein from smooth muscle, and showed that its distribution within cultured fibroblasts was nearly identical to

FIGURE 5 Radioimmunoassay for vinculin (V), the 135-kD protein (135), and talin (T) in extracts of chicken heart, lens, and gizzard. The tissue extracts at serial 1:3 dilutions were immobilized on the surface of microtiter wells and an indirect binding assay was carried out (see Materials and Methods). Notice the comparable concentrations of all three proteins in heart as compared to the relatively low levels of talin in lens and of the 135-kD proteins in gizzard.

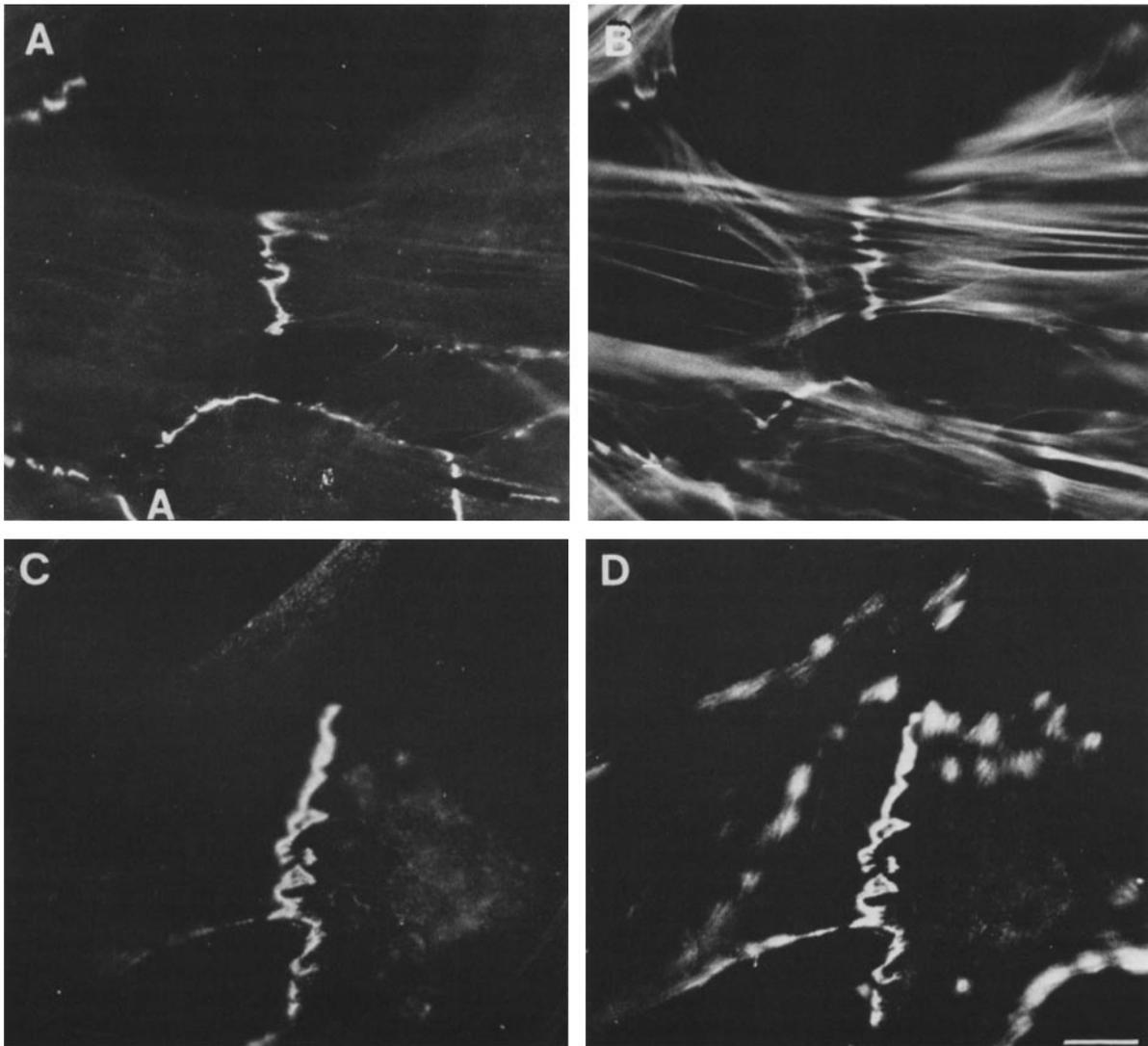


FIGURE 6 Double-fluorescent localization of the 135-kD protein (A and C) and actin (B) or vinculin (D) in cultured chicken lens cells. Notice that actin bundles terminate at the 135-kD protein-rich junctions and the apparent absence of labeling for the latter from vinculin-rich cell-substrate focal contacts. Bar, 10 μ m.

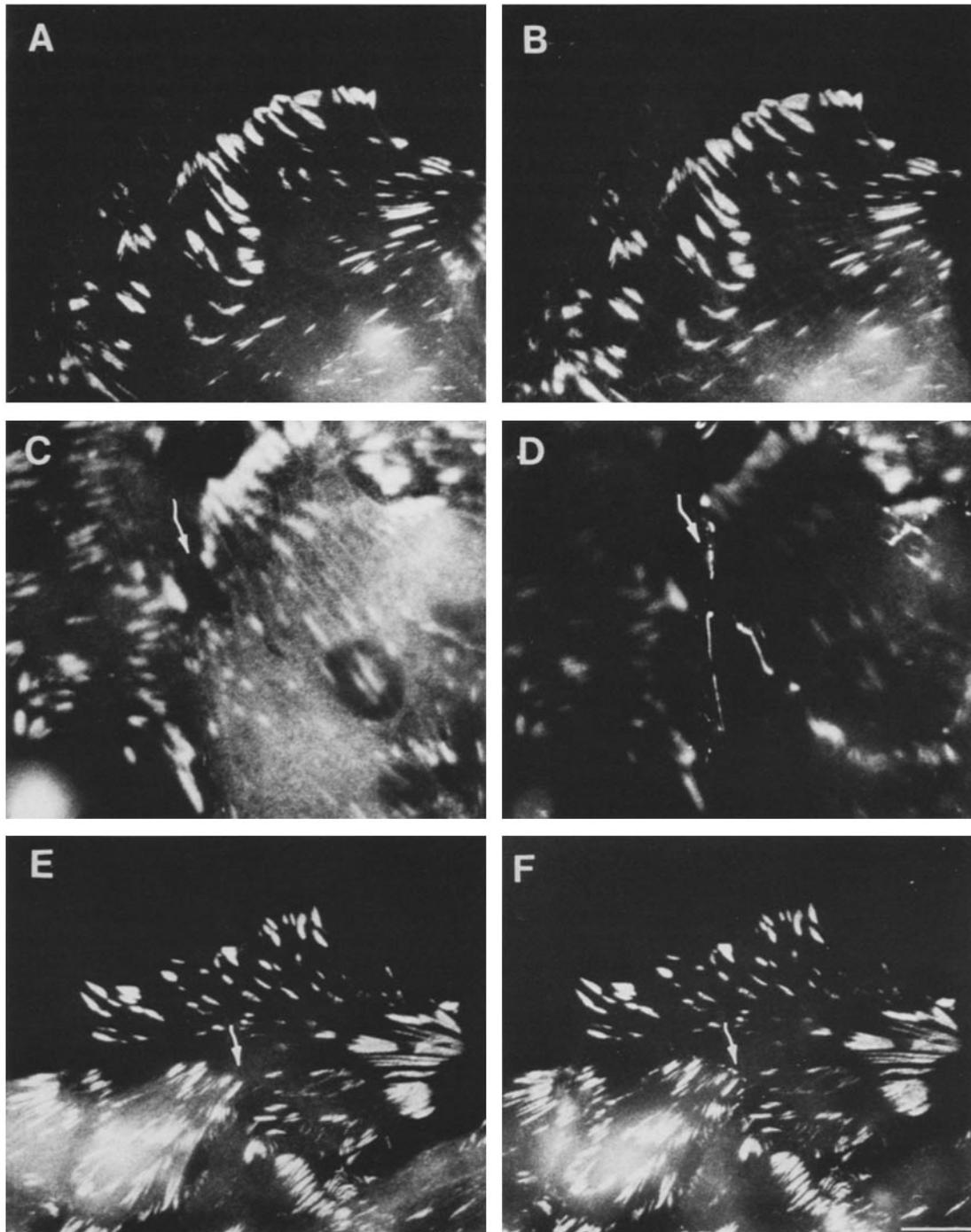


FIGURE 7 Double-immunofluorescent localization of talin (A, C, and E) and vinculin (B, D, and F) in cultured chicken lens cells at different cell densities. In individual cells, the labeling for the two is nearly identical, whereas in dense areas it is apparent that talin is absent from the spotty or extended intercellular, vinculin-rich junctions (see arrows in C-F). Bar, 10 μ m.

that of vinculin (9, 10). Selective removal of membrane-bound actin by fragmin as described above followed by immunofluorescent labeling for talin suggested that the latter, like vinculin, was a component of the junctional plaque. A different junctional component is the 135-kD membrane protein, which we have reported to be present along intercellular adherens junctions facing the cell exterior (45). The detailed molecular properties of the 135-kD protein are still poorly characterized. It is noteworthy however that another molecule of somewhat lower molecular weight, i.e., uvomorulin,

was localized by immunogold labeling in the intercellular gap of intestinal *zonula adhaerens* (6). The relationships between the 135-kD protein and uvomorulin are currently under investigation.

In this study we have shown that adherens junctions are molecularly heterogeneous and may be subdivided according to their molecular constituents into two major subfamilies. Those formed with neighboring cells contain vinculin and the 135-kD protein, but apparently no talin, whereas contacts with noncellular materials contain talin and vinculin only.

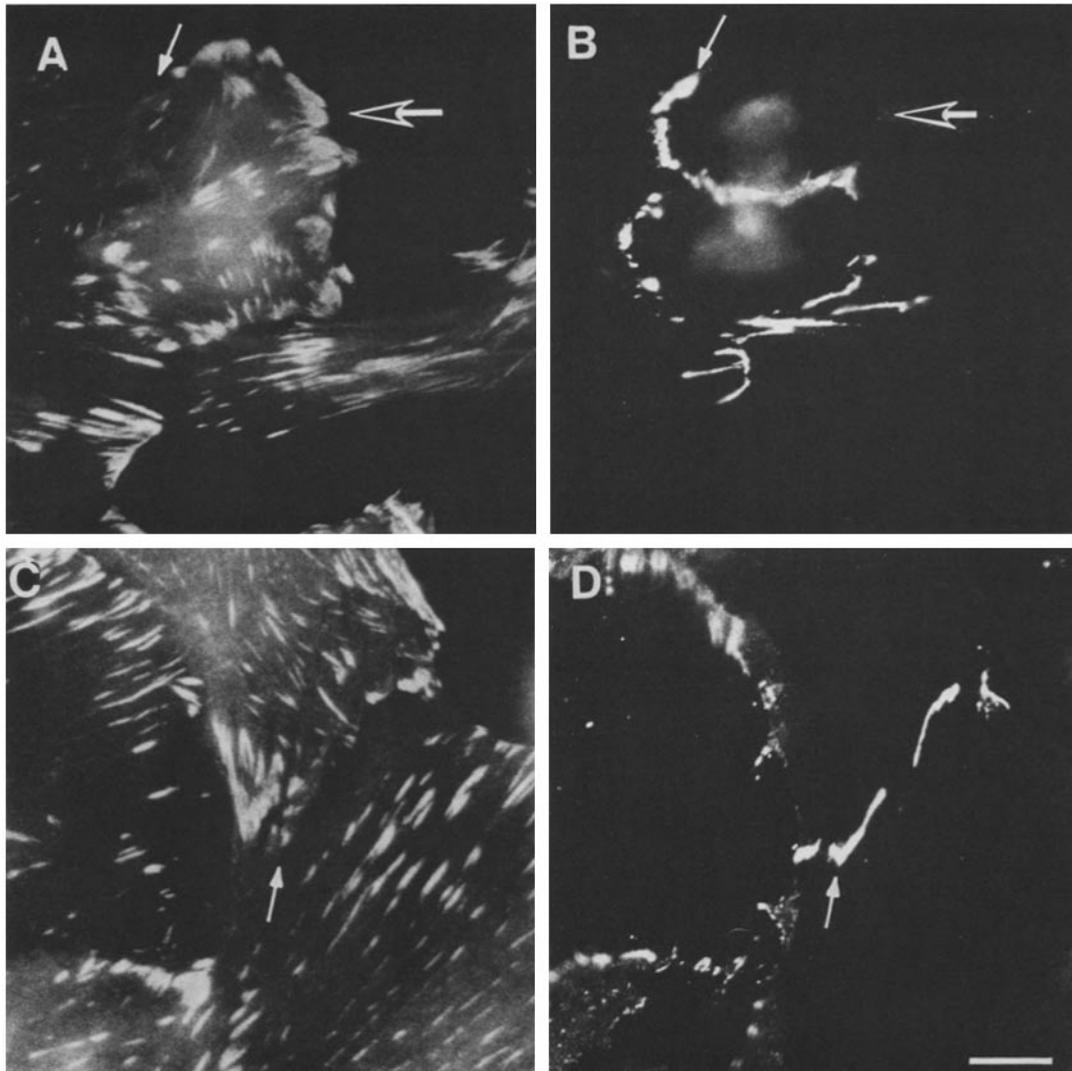


FIGURE 8 Double-immunofluorescent labeling of the same cultured chicken lens cells for talin (A and C) and the 135-kD protein (B and D). The immunofluorescent patterns of distribution of the two proteins are mutually exclusive. The solid arrows point to the 135-kD-containing intercellular junctions which are devoid of talin; the empty arrow points to talin-rich focal contact. Bar, 10 μ m.

This distinction holds both for intact tissues and for cultured cells. The differences in distribution within tissues were most clearly apparent by immunofluorescent labeling of intact chicken lens and chicken gizzard. The lens tissue contains alternating areas of gap junctions and intercellular adherens junctions. Electron microscopy of this tissue together with fluorescence microscope examinations (Fig. 4) pointed to the abundance of actin, vinculin, and the 135-kD protein along the membrane. Smooth muscle cells of the gizzard, on the other hand, are usually interspaced by connective tissue and apparently form only few direct intercellular contacts. Accordingly, they contain only vinculin and talin along their membrane-bound dense plaques. Crude semi-quantitative estimation of the content of the three junctional proteins (vinculin, talin, and the 135-kD protein) by an antibody binding assay, suggested that their relative levels of expression were generally comparable to the intensities of the immunofluorescent labeling. The amounts of the 135-kD protein in gizzard were low as compared to lens or to heart, and talin was present in relatively small quantities in lens tissue. It should be pointed

out that the low levels of the 135-kD protein and talin detected in gizzard and lens, respectively, represent an actual presence of the two proteins and are not due to nonspecific background. In view of the immunofluorescent results it seems likely that the 135-kD protein in gizzard and talin in lens are present in nonjunctional compartments, and may become associated with the plasma membrane only after new types of cell contacts are established as probably happens under culture conditions. It still remains to be determined at what level and by which mechanisms the expression of these proteins is regulated in the intact tissues.

It is, however, evident from the present studies that the distinct subcellular distributions of talin and the 135-kD protein may be also spatially regulated within individual cells, irrespective of their synthesis. Thus, in the same cultured lens cells, both proteins may be present, displaying mutually exclusive association with either focal contacts or with intercellular junctions. This finding of selective spatial regulation is especially interesting in view of the known biochemical interactions between adherens junction components. It has been

directly shown that vinculin can bind to actin (33, 46) and to a ~200-kD protein, identified as talin (11, 36). It is, therefore, possible that the selective exclusion of talin from the vinculin plaques of cell-cell contacts involves either the presence of different forms of vinculin in the two subfamilies of junctions with distinct capacity to bind talin or a selective segregation of other proteins (yet unidentified) in these junctional complexes. Attempts to define the extent of molecular heterogeneity and to identify other junctional components either common or junction-specific are presently in progress.

The molecular differences between cell-cell and cell-matrix contacts reported here may also bear on the involvement of the two in physiological cellular activities. Generally, cell-substrate adhesions are believed to be essential for cell motility and growth (phenomena often referred to as anchorage dependence [for review see references 43 and 44]) while extensive cell-cell contacts typical of confluent cultures lead to an arrest of motility and growth, namely contact paralysis and contact inhibition (1, 2, 28, 30). There is still no direct evidence indicating that these two markedly different types of contact-induced transmembrane signals are transmitted specifically through the two corresponding subfamilies of adherens junctions described here. This possibility seems, however, attractive in view of the correlation between loss of anchorage dependence and contact-inhibition in transformed cell and the concomitant generalized deterioration of adherens junctions (for additional discussion of this aspect see reference 21). The findings reported here on molecular differences between the two types of contacts and a better understanding of their fine and detailed architecture may provide us with valuable information regarding basic mechanisms involved in the control of cell growth and motility.

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