

## A 135-kd membrane protein of intercellular adherens junctions

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Communicated by B.Geiger

**We report here on a new 135-kd membrane protein which is specifically associated with intercellular adherens-type junctions. This surface component was identified by a monoclonal antibody, ID-7.2.3, raised against detergent-extracted components of membranes of chicken cardiac muscle rich in intercalated discs. The antibodies stain extensively adherens junctions in intact cardiac muscle and in lens, as well as in cultured cells derived from these tissues. In living cultured cells only very little immunolabelling was obtained with ID-7.2.3 antibodies, probably due to the limited accessibility of the antibodies to the intercellular gap. However, upon the removal of extracellular  $\text{Ca}^{2+}$  ions a dissociation of the junction occurred, leading to the rapid exposure of the 135-kd protein. Immunoelectron microscopic labelling of EGTA-treated, or detergent-permeabilized cells indicated that the antigen is found along the plasma membrane and highly enriched in contact areas. Double immunolabelling for both the 135-kd protein and vinculin pointed to the close association of the two in intercellular junctions and to the apparent absence of the former protein from the vinculin-rich focal contacts of cultured cells and from dense plaque of smooth muscle. Immunoblotting indicated that the 135-kd protein is present in many tissues but is particularly enriched in heart, lens and brain.**

**Key words:** adherens junctions/membrane protein/cardiac muscle/lens/vinculin

### Introduction

Cells in multicellular organisms often form stable junctions with their neighbours or with elements of the surrounding extracellular matrix (ECM). Many of the structural features of such junctions and hemi-junctions have been documented but their detailed molecular organization is still poorly known (for reviews, see Farquhar and Palade, 1963; Gilula, 1974; Staehelin, 1974; Grinnell, 1978; Goodenough, 1980; Aplin and Hughes, 1982).

Of the four to five major types of intercellular junctions (Staehelin, 1974) two are known to be associated, at their cytoplasmic aspects, with cytoskeletal filaments. Adherens junctions and their ECM- or substrate-attached counterparts are associated with actin bundles through vinculin-containing, membrane-bound plaques (Geiger *et al.*, 1980, 1983; Geiger, 1981, 1982), while desmosomes are associated with tonofilaments, mostly of the cytokeratin class (Kelly, 1965; Campbell and Campbell, 1971; Franke *et al.*, 1982; Geiger *et al.*, 1983; Kartenbeck *et al.*, 1983).

Several experiments have suggested that adherens-type junctions contain at least three distinct structural domains: (i) an integral membrane domain which contains specific

'contact receptors'; (ii) a membrane-bound cytoplasmic plaque which contains vinculin, talin and possibly additional 'peripheral' proteins; (iii) a cytoplasmic bundle of microfilaments consisting of actin and several associated proteins, which bind to the membrane-associated plaque. It had been proposed that contact-induced immobilization of the 'contact receptors' leads to the transmembrane attachment of vinculin to the nascent contact area and subsequently to the initiation of actin bundle assembly (Geiger, 1981, 1982). Attempts to identify 'contact receptors' in junctional membranes and to isolate them have not however been successful.

We report here the identification of a 135-kd membrane protein which is specifically associated with intercellular adherens junctions in cardiac muscle, lens tissue and other organs, as well as in cell cultures from these tissues. This newly described protein was detected with a specific monoclonal antibody prepared against amphipathic proteins of chicken cardiac muscle intercalated discs.

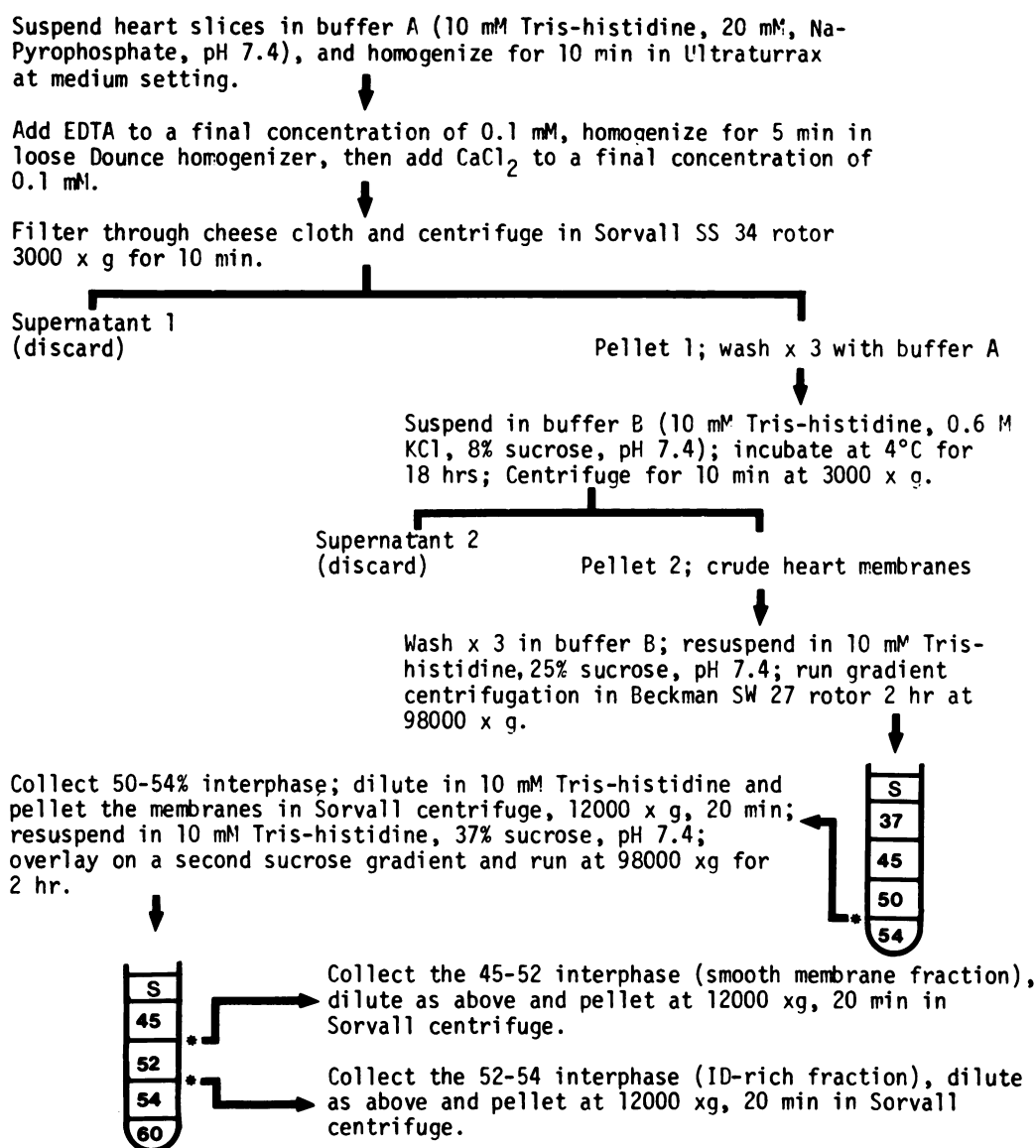
### Results

#### *Isolation of amphipathic proteins from chicken cardiac muscle intercalated discs*

Intercalated discs of chicken cardiac muscle were isolated after homogenization of the tissue, extraction with high salt buffer and sucrose gradient centrifugations as outlined in Figure 1; the procedure is similar to that described by Colaco and Evans (1982). After the two sucrose gradient centrifugations we obtained two major membrane-rich fractions; one collected at the 45–52% sucrose interphase (P45–52) and the other collected at the 52–54% sucrose interphase (P52–54). Transmission electron microscopy indicated that the former consisted predominantly of smooth membrane fragments and vesicles (Figure 2a) while the latter was enriched with intercalated discs (arrows in Figure 2b). As the figure shows, in addition to intercalated discs (ID), P52–54 contained non membrane material, mostly residues of myofibrillar filaments.

Membrane constituents from the ID-rich fraction were isolated by Triton X-114 extraction and subsequent detergent partitioning at 37°C. The isolated membranes were extracted at 0°C with the detergent, the insoluble residue removed by centrifugation and the extract warmed to 37°C (for details see Materials and methods). [Previous experiments have indicated that proteins would usually partition into the two phases according to their water solubility. Thus, integral membrane proteins which are embedded in the lipid bilayer and apparently expose on their surface hydrophobic domain(s) are found in the detergent phase while peripheral or 'cytosolic' proteins partition into the upper buffer phase (e.g., Bordier, 1981; Coudrier *et al.*, 1983).]

Analysis of the different fractions by SDS-PAGE revealed significant differences between the P45–52 and P52–54 fractions enriched with smooth membranes or with ID, respectively. The peptides were visualized by either Coomassie blue staining (Figure 3a–c), or by [ $^{125}\text{I}$ ]Con-



**Fig. 1.** Purification of ID-rich membranes from chicken cardiac muscle. In regular purifications we have used 30–50 g of cardiac muscle. The homogenization and subsequent extractions were carried out with 10 volumes of the respective buffers. The numbers listed in the centrifuge tubes refer to the concentration of sucrose (w/v); S, sample. The fractions collected are marked with asterisks.

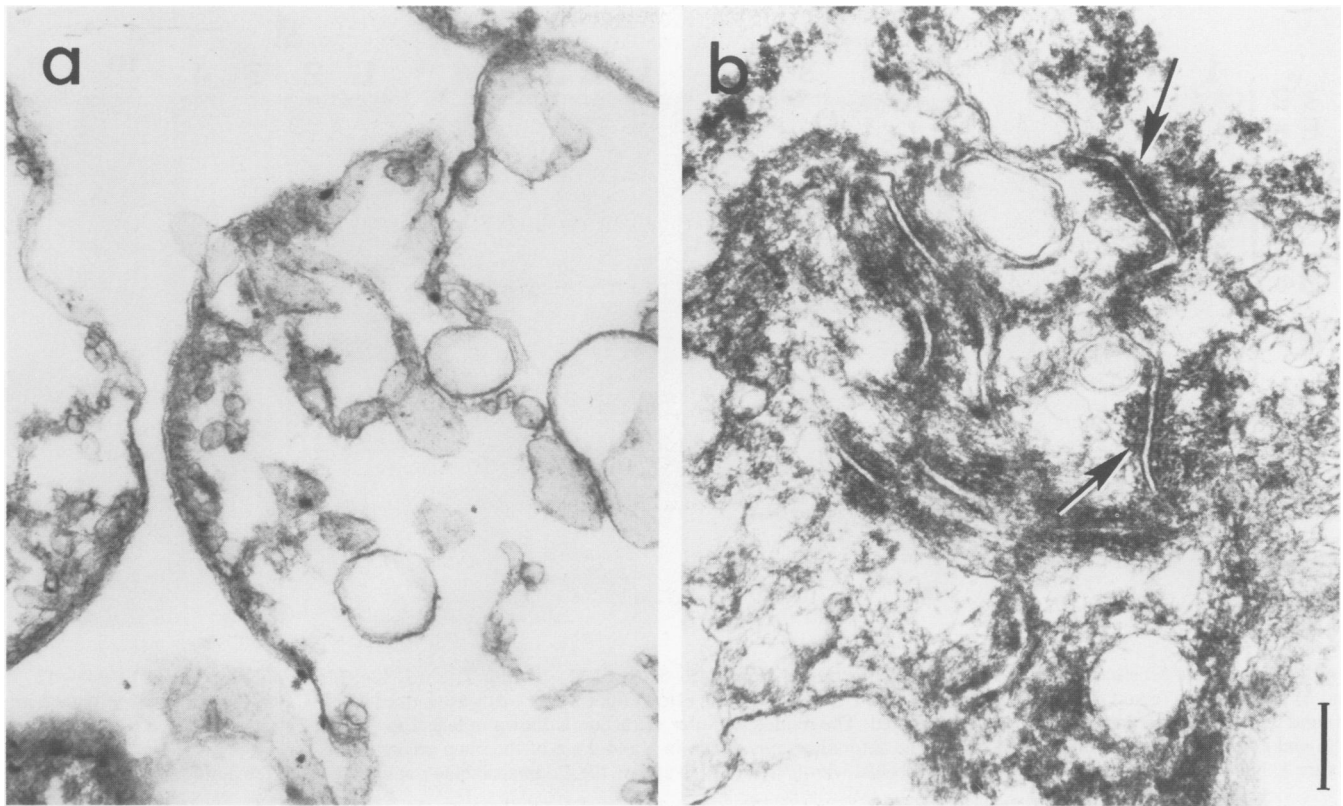
canavalin A (Con A) overlay to detect mannose-containing glycoproteins (Figure 3a'–c').

The extraction of proteins from P45–52 and P52–54 by Triton X-114 and their segregation into different phases is shown in Figure 4. There are polypeptides shared by the detergent extracts of P45–52 (a and b) and P52–54 (c and d). However, the Con A-binding glycoproteins of the two fractions were significantly different (compare lanes 1b and 1d in Figure 4). Beside glycoprotein with a mol. wt. of ~95 kd, shared by P45–52 and P52–54, the extract of the latter fraction contained additional glycoproteins in the range between 200 kd and 37 kd. Moreover, P52–54 contained considerably more detergent-insoluble proteins (lanes 2A and 2C) and glycoproteins (lanes 2b and 2d). The major glycoproteins (95 kd, 50–55 kd, 120–140 kd, etc.) were minor protein components in the P52–54 fraction (compare the [ $^{125}\text{I}$ ]Con A labelled bands to the corresponding Coomassie blue-stained lanes). The specificity of the Con A labelling is shown by its exclusive reaction with ovalbumin out of the six marker proteins used. [Compare the Coomassie blue pattern

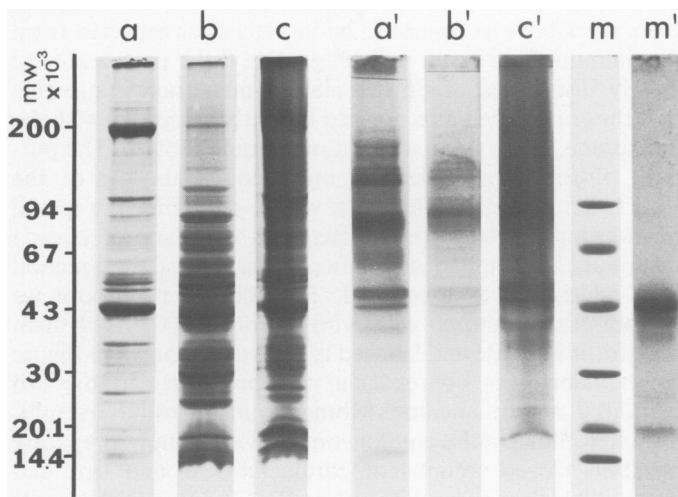
(m) with the [ $^{125}\text{I}$ ]Con A labelling (m').] Vinculin was highly enriched in the P52–54 fraction as compared to P45–52. This was verified by immunoblotting the various fractions using anti vinculin monoclonal antibodies (not shown).

#### *Monoclonal antibody ID-7.2.3*

Mice were injected with the detergent phase of the Triton solubilized, ID-containing P52–54 fraction. After fusion we selected reactive clones by two consecutive assays: (i) differential solid-phase radioimmunoassay with the detergent phases of P52–54 and P45–52 and (ii) immunofluorescent labelling of cardiac tissue and cultured cells with the different hybridoma supernatants. Of 21 positive hybridomas, four cultures produced antibodies preferentially reacting with the P52–54 fraction and only poorly with P45–52. One of these lines, namely ID-7.2, stained positively and extensively IDs of chicken cardiac muscle (Figure 5a, compare with the phase contrast photomicrograph in b). This hybridoma line was then cloned in soft agar and clone No. 3 propagated in culture and in ascites form.



**Fig. 2.** Transmission electron photomicrograph of the P45–52 (a) and P52–54 (b). Notice that the former fraction contains mostly smooth membranes while the latter is highly enriched with intercalated discs (two of which are marked with arrows).



**Fig. 3.** Polyacrylamide gel electrophoresis of: a and a' crude heart membranes (pellet 2 in Figure 1), b and b': P45–52, c and c': P52–54. The gels were labelled with either Coomassie blue (a, b, c, m) or with [ $^{125}$ I]Con A (a', b', c', m'). The markers used were (from top to bottom) phosphorylase b, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor,  $\alpha$ -lactalbumin. Notice that among the marker proteins only ovalbumin is labelled with [ $^{125}$ I]Con A.

#### *Monoclonal antibodies ID-7.2.3 bind to intercellular contacts*

Immunofluorescent labelling with ID-7.2.3 was carried out on chick heart, liver, gizzard, lens, tongue and intestine. The tissues were fixed with 3% paraformaldehyde and frozen sections of either 3–4  $\mu$ m or 0.5–1  $\mu$ m were cut.

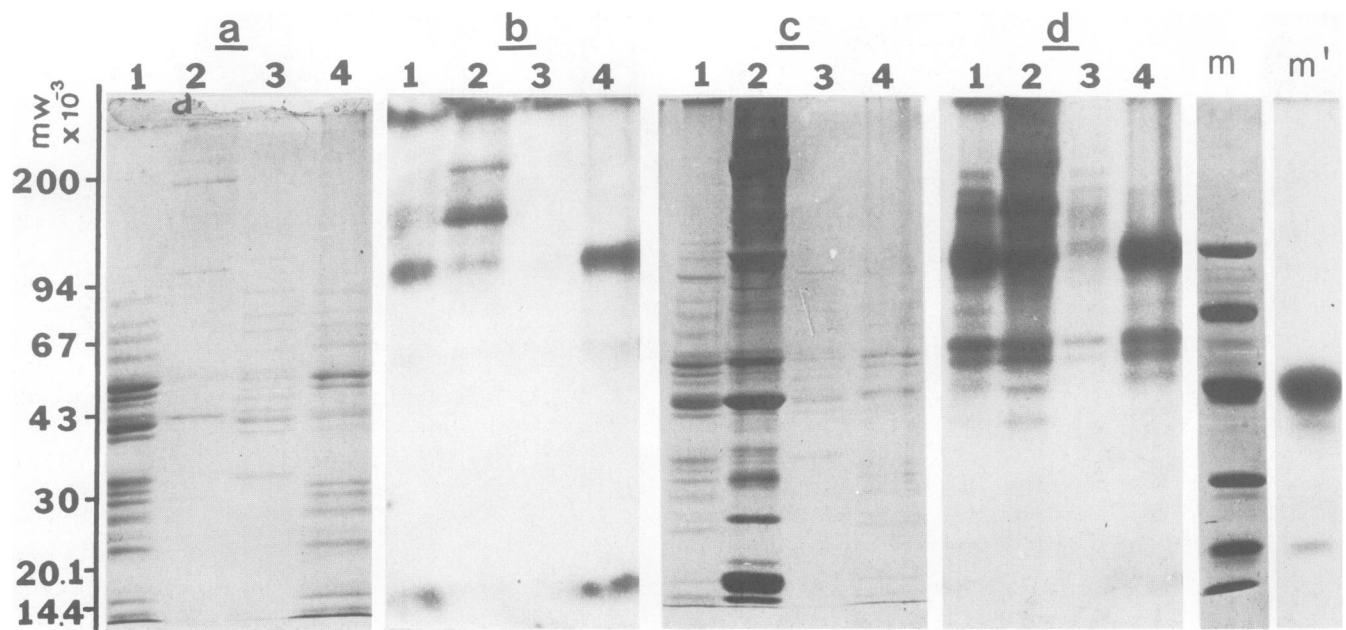
As pointed out above, in cardiac muscle extensive labelling of the intercalated discs was observed as well as labelling of

numerous patches scattered along the lateral borders of the cells. These lateral patches often showed periodic arrangement (see bars at the top of Figure 5a) matching the periphery of the z discs of the muscle (see matching bars in Figure 5b). Very extensive labelling with ID-7.2.3 was also obtained in frozen sections of chick lens (Figure 5c). Here the labelling was almost continuous along the entire plasma membranes, as seen in both longitudinal and cross-sections of lens fibers. Liver, gizzard and tongue tissues labelled poorly and its significance is not clear. Faint labelling was observed in the subapical regions of intestinal epithelium; this labelling could be intensified by short exposure of 1  $\mu$ m sections to 0.1% Triton X-100 but the structural preservation of the specimens after the detergent extraction was not satisfactory.

Cultured cells, which were briefly extracted with Triton X-100 and fixed with formaldehyde exhibited extensive labelling with ID-7.2.3 along contacts formed between them. In cardiac cells the labelling was associated with elongated patches, often spanning the entire contact region (Figure 6a). Attachment in neighbouring fibroblasts, or in cultured gizzard cells, was usually mediated by numerous thin membrane protrusions which were stained by ID-7.2.3 mAbs (Figure 6b). Very extensive labelling was noted in cultures of chick lens cells (Figure 6c) or of chick pigment epithelium (Figure 6d). The labelling was continuous along the subapical intercellular junctions of these polarized cells. It should be emphasized that in all cells tested no labelling was detected near focal contacts formed between the ventral surfaces of the cells and the substrate (see below).

#### *Monoclonal antibodies ID-7.2.3 bind to a membrane component of intercellular adherens junctions*

The above results indicated that ID-7.2.3 antibodies bind to a



**Fig. 4.** Polyacrylamide gel electrophoresis of polypeptides extracted from P45–52 and P52–54 with Triton X-114. Membrane suspensions of P45–52 (**a** and **b**) and P52–54 (**c** and **d**) were extracted with 1% Triton X-114. The extract (lane 1 in **a–d**) was warmed to 37°C and the buffer phase (lane 3 in **a–d**) and detergent phase (lane 4 in **a–d**) were collected. The residue left after extraction is shown in lane 2 (**a–d**). The gels were stained with Coomassie blue (**a** and **c**) and subsequently with [ $^{125}$ I]Con A. The autoradiograms shown in **b** and **d** are of the same gels as **a** and **c**, respectively. Markers are as detailed in Figure 3. Note that only ovalbumin is positively labelled with [ $^{125}$ I]Con A [compare the Coomassie blue staining (**m**) with the autoradiogram (**m'**)].

junctional component but they did not point to the exact cellular domain containing the antigen, nor did they define the type of junction with which it was associated.

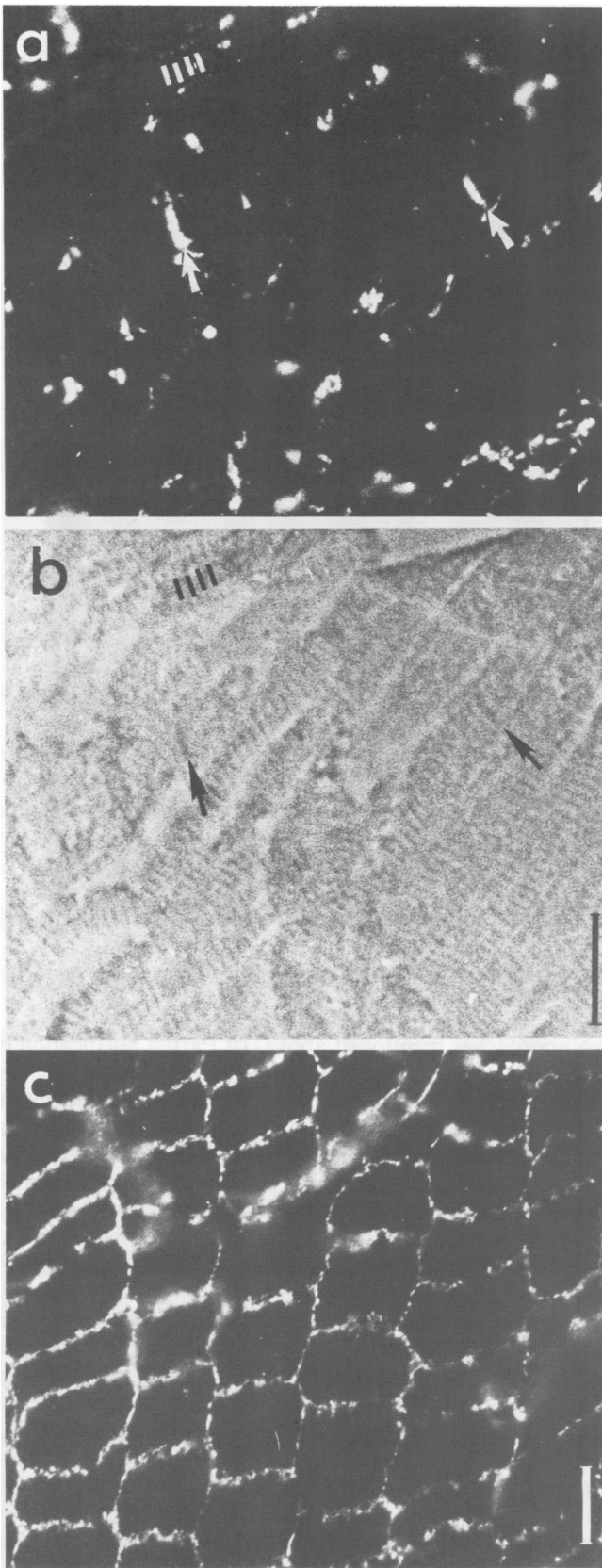
Several lines of evidence (see below) suggested that the antigen was closely associated with the junctional plasma membrane and that the specific epitope of mAb ID-7.2.3 was facing the exterior of the cells. Living, or formaldehyde-fixed, cardiac cells in cultures, which were not permeabilized, exhibited very low levels of labelling with ID-7.2.3 antibodies as compared with detergent-permeabilized cells (Figure 7a). One possible explanation was the masking of the epitope in the narrow intercellular gap. To separate the neighbouring cells and expose the epitope we incubated cultured cardiac cells with EGTA. After short incubation with 4–5 mM EGTA the junction was split-open and the exposed component(s) in the junctional membranes became accessible to the antibody (Figure 7b and c). After longer periods (5–10 min) separation of the two halves of the junction was complete. Later, it became increasingly difficult to detect the residual half junctions, possibly due to lateral diffusion of the antigen from its original location on the junctional membrane.

In fixed cultured lens cells, the immunolabelling along the junctions was weak though more extensive than in cardiac cells. Nevertheless incubation with EGTA brought about remarkable enhancement in labelling of the junctions (Figure 7, compare the fixed cells in e with the EGTA-treated cells in f–h). Interestingly, after longer incubation, for 10 min or more, the epitope of ID-7.2.3 mAbs diffused laterally from the lens junctions and the overall labelling of the cell surface became uniform (Figure 7h). The EGTA treatment did not permeabilize the cells since no immunolabelling was found in the same cells when incubated with actin or vinculin antibodies.

The antigen was further localised in the plasma membrane by immunoelectron microscopy. Cultures of cardiac cells were treated with EGTA for 10 min, then fixed and immuno-

labelled indirectly with ID-7.2.3 mAbs and gold-labelled goat anti-mouse F(ab')<sub>2</sub>. As Figure 8a and b indicate extensive immunogold label is present on the surface of some of the cells, in agreement with the immunofluorescent data. In some cases the label was concentrated in patches as expected from the immunofluorescent labelling. The gold particles were closely distributed along the plasma membrane suggesting that the antigen being recognized is tightly associated with the membrane, possibly an integral membrane protein. The purpose of the immunoelectron microscopic labelling of the EGTA-treated cells at this stage was to determine the spatial relationships between the antigen and the plane of plasma membrane and not to determine the nature of the junction with which it was associated. For the latter purpose we permeabilized cultured cells with Triton X-100, fixed them with formaldehyde and labelled as above. As shown in Figure 8c the label was concentrated predominantly at the cell periphery, near filamentous submembranal densities resembling intercellular adherens junctions. We are now preparing ultrathin frozen sections of cardiac muscle and lens and immunolabelling them with mAbs ID-7.2.3 (see Discussion).

The antigen-labelled by ID-7.2.3 was clearly not associated with desmosomes; double labelling of cultured cardiac cells with both desmin antibodies and ID-7.2.3 indicated that the antigen labelled by the latter was excluded from desmin rich areas at the cell periphery and *vice versa* (Figure 9 c–f). In cardiac cells desmin is associated with the desmosomes or *maculae-adherens* (Kartenbeck *et al.*, 1983). Moreover, we have often observed junctions between presumptive muscle cells (desmin-positive) and non-muscle cells (desmin-negative), both of which were positively labelled with ID-7.2.3 mAbs (Figure 9e and f). On the other hand, double labelling of the same cardiac cells with ID-7.2.3 mAbs and anti-vinculin gave a pattern of partial identity (Figure 9a and b). Both antibodies labelled extensively the same intercellular contact areas while only vinculin was detected near the



**Fig. 5.** Immunofluorescent labelling of thin (0.5  $\mu\text{m}$ ) frozen sections of chicken cardiac muscle (**a**) and chick eye-lens (**c**). The phase contrast photomicrograph in **b** is of the same area of cardiac muscle shown in **a**. Matching arrows in **a** and **b** point to the same intercalated discs. The array of bars points to positively labelled periodic patches along the lateral cell borders corresponding to the periphery of the z discs. Bars in **b** and **c** represent 10  $\mu\text{m}$ .

substrate-associated focal contacts (arrowhead in Figure 9a and b).

The close spatial relationships between the antigen labelled with ID-7.2.3 and vinculin were clear in cultured lens cells. Figure 10 shows that the subapical junctions were positively labelled with both ID-7.2.3 (**a**) and anti-vinculin (**b**). At the ventral focal plane of exactly the same group of cells vinculin-containing focal contacts were apparent (Figure 10c) with no corresponding labelling with ID-7.2.3. This is further shown in Figure 10d and e, of a single, well-spread cell with many vinculin containing focal contacts (**e**) and essentially no labelling with ID-7.2.3 antibodies (**d**). For these and other reasons to be discussed we suggest that the antigen detected by ID-7.2.3 mAbs is specifically associated with vinculin-rich intercellular adherens junctions of these tissues.

*The junctional component detected by ID-7.2.3 mAbs is a 135-kd membrane protein*

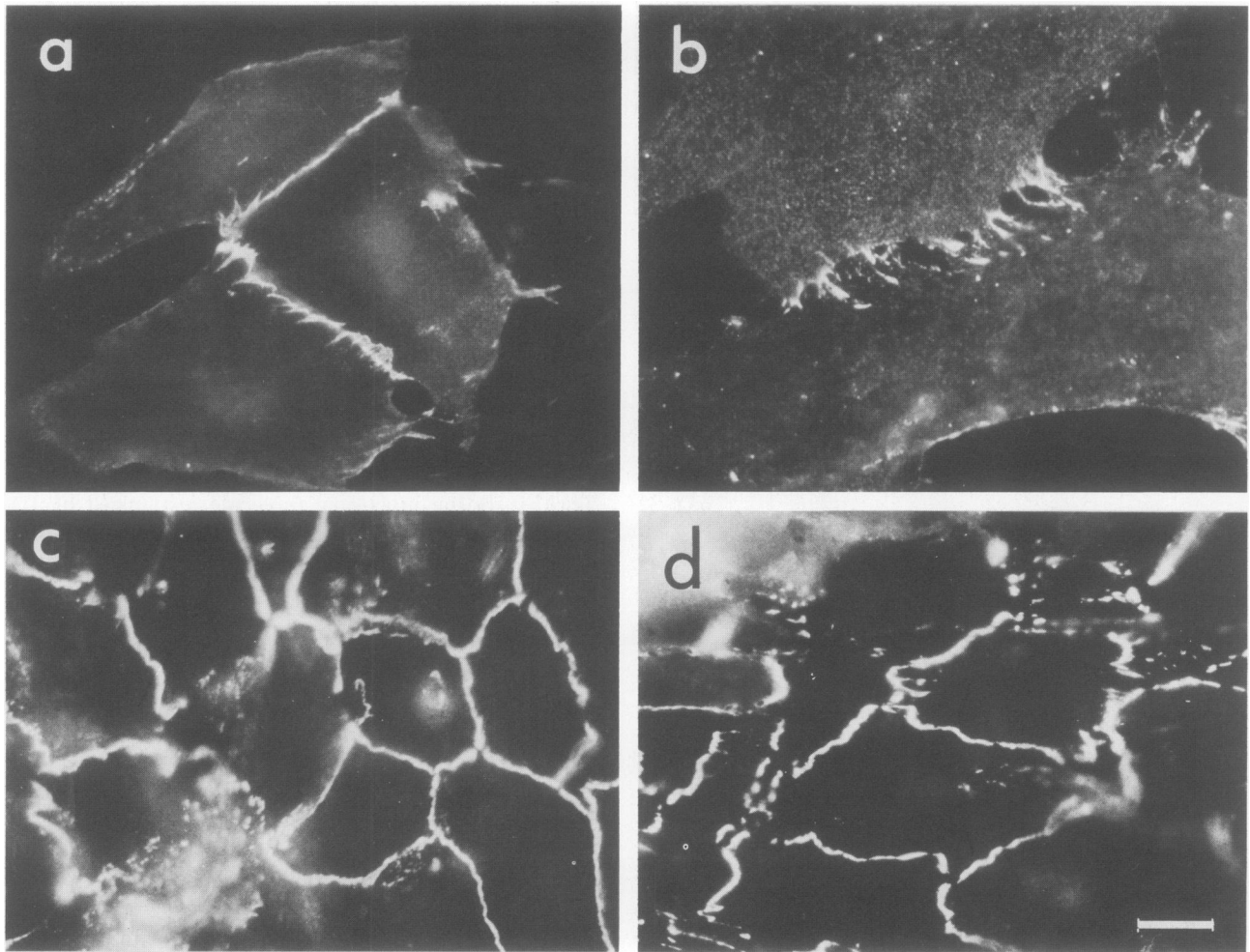
To identify the junctional molecule recognized by ID-7.2.3 antibodies we performed immunoblotting analysis of freshly excised samples of several chick tissues. The results (Figure 11) indicated that a polypeptide with mol. wt. of  $\sim 135\,000$  was specifically labelled in all the tissues tested. The highest relative concentrations of the protein (as estimated from the intensity of the bands on the autoradiogram) were found in cardiac muscle and in lens, in line with the immunocytochemical results. Attempts to identify the 135-kd protein in the various fractions of chick cardiac muscle or in the detergent extract indicated that the antigen is sensitive to proteolysis and is progressively degraded during isolation of intercalated discs and their extraction with detergents. Further biochemical and immunochemical characterization of the 135-kd protein is in progress.

## Discussion

The major classes of junctions including gap junctions, tight junctions, desmosomes and adherens junctions are usually identified by their distinctive morphology and little is known about their detailed molecular structure. Attempts have been made to identify molecules involved in cell-contact formation, including those present in defined cellular junctions, using two major experimental approaches: (i) the isolation of specific cell-contacts and the biochemical or immunochemical characterization of their components; (ii) the production of antibodies (multispecific or monoclonal) which perturb cell contact formation and the identification of the cellular constituents recognized by these antibodies.

The former approach was used to identify the constituents of gap-junctions (Goodenough and Stoeckenius, 1972; Duguid and Revel, 1976; Culvenor and Evans, 1977; Ehrhart and Chauveau, 1977; Handerson *et al.*, 1979; Herzberg and Gilula, 1979; Finbow *et al.*, 1980) and recently of epidermal desmosomes (Gorbsky and Steinberg, 1981; Franke *et al.*, 1982; Cohen *et al.*, 1983; Kartenbeck *et al.*, 1983; Mueller and Franke, 1983). These two intercellular junctions are abundant in certain tissues (liver or lens for the former and epidermis for the latter) and exhibit remarkable stability which enabled their isolation in essentially pure form. Similar attempts to purify tight junctions or adherens junctions have not yet been successful, possibly due to their lability and relatively low abundance.

Antibodies which affect cell adhesion enabled the identification of different adhesion-related molecules though their specific association with defined junctions is still not clear



**Fig. 6.** Indirect immunofluorescent labelling of cultured cells with ID-7.2.3 mAbs. The cells were permeabilized, then fixed and labelled. **a:** chicken cardiac myocytes **b:** chicken gizzard cells **c:** chick eye lens cells **d:** chick pigment epithelium of the retina. Bar represents 10  $\mu$ m.

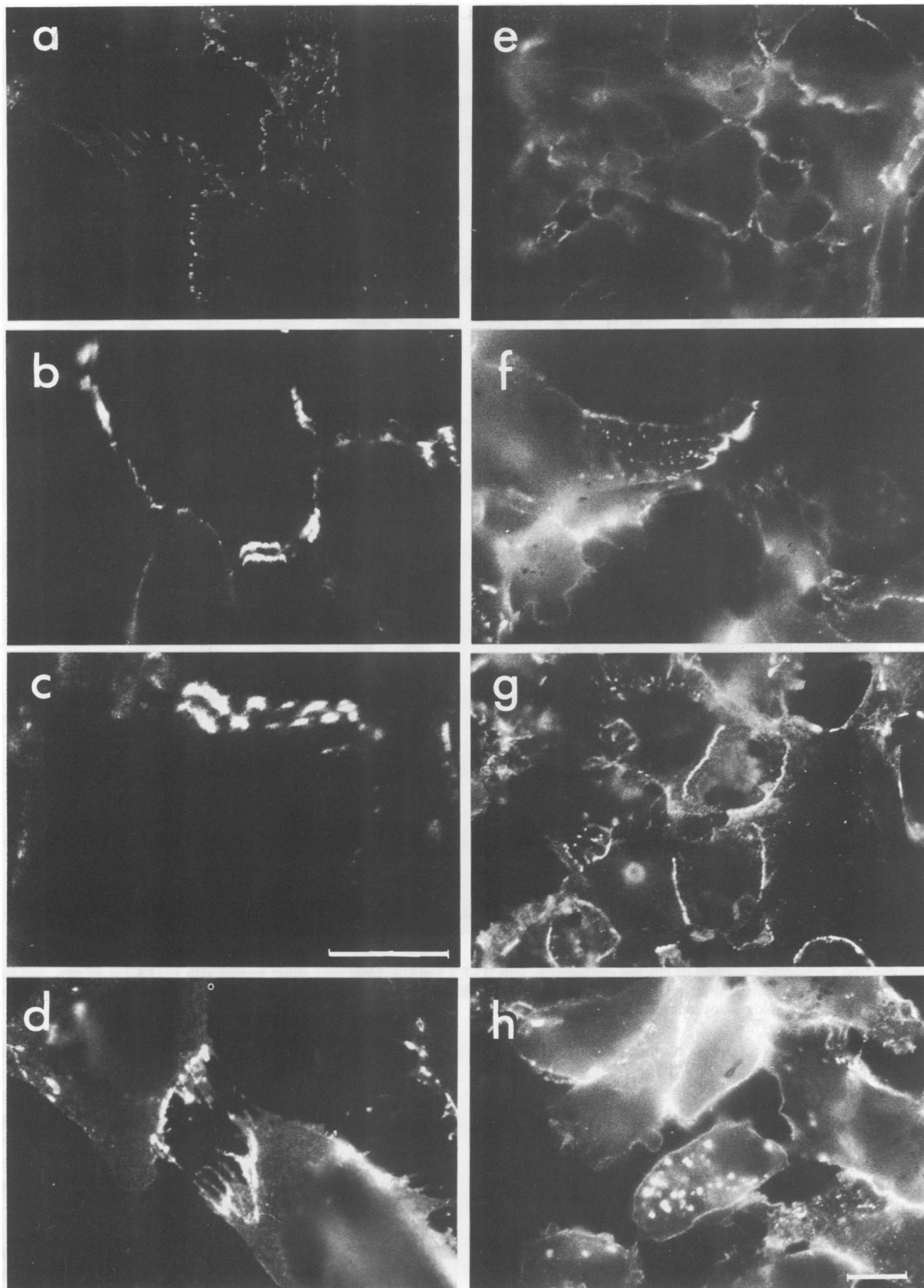
(e.g., McClay and Moscona, 1974; Brachenburry *et al.*, 1977; Thiery *et al.*, 1977; Rutishauser *et al.*, 1978a, 1978b; Urushihara and Takeichi, 1980; Knudsen *et al.*, 1981; Oesch and Birchmeier, 1982; Imhof *et al.*, 1983).

Here we have tried to identify specific molecular constituents of adherens junctions. As pointed out earlier (Geiger *et al.*, 1983), the 'classical' adherens junctions, such as *zonula adherens* of polarized epithelia or *fascia adherens* of cardiac muscle are belt-like, or patchy intercellular contacts to which microfilaments are attached through electron-dense plaques. In frozen-etched samples these junctions show no distinctive organization of intramembrane particles and their electron microscopic definition is often difficult. Our molecular definition for adherens junctions was based on immunocytochemical labelling for actin and several associated proteins. In particular the cytoskeletal protein vinculin was typical of adherens junctions (Geiger *et al.*, 1980, 1983; Geiger, 1981, 1982). *In vitro* studies indicate that vinculin binds to both actin and to the junctional plasma membrane (Jockusch and Isenberg, 1981, 1982; Isenberg *et al.*, 1982; Wilkins and Lin, 1982; Avnur *et al.*, 1983) and may therefore be involved in the linkage between the two. Vinculin was detected in the typical adherens junctions as well as in cell-matrix attachments in tissues and in cultured cells (for an extensive discussion see Geiger *et al.*, 1980; Geiger, 1981, 1982).

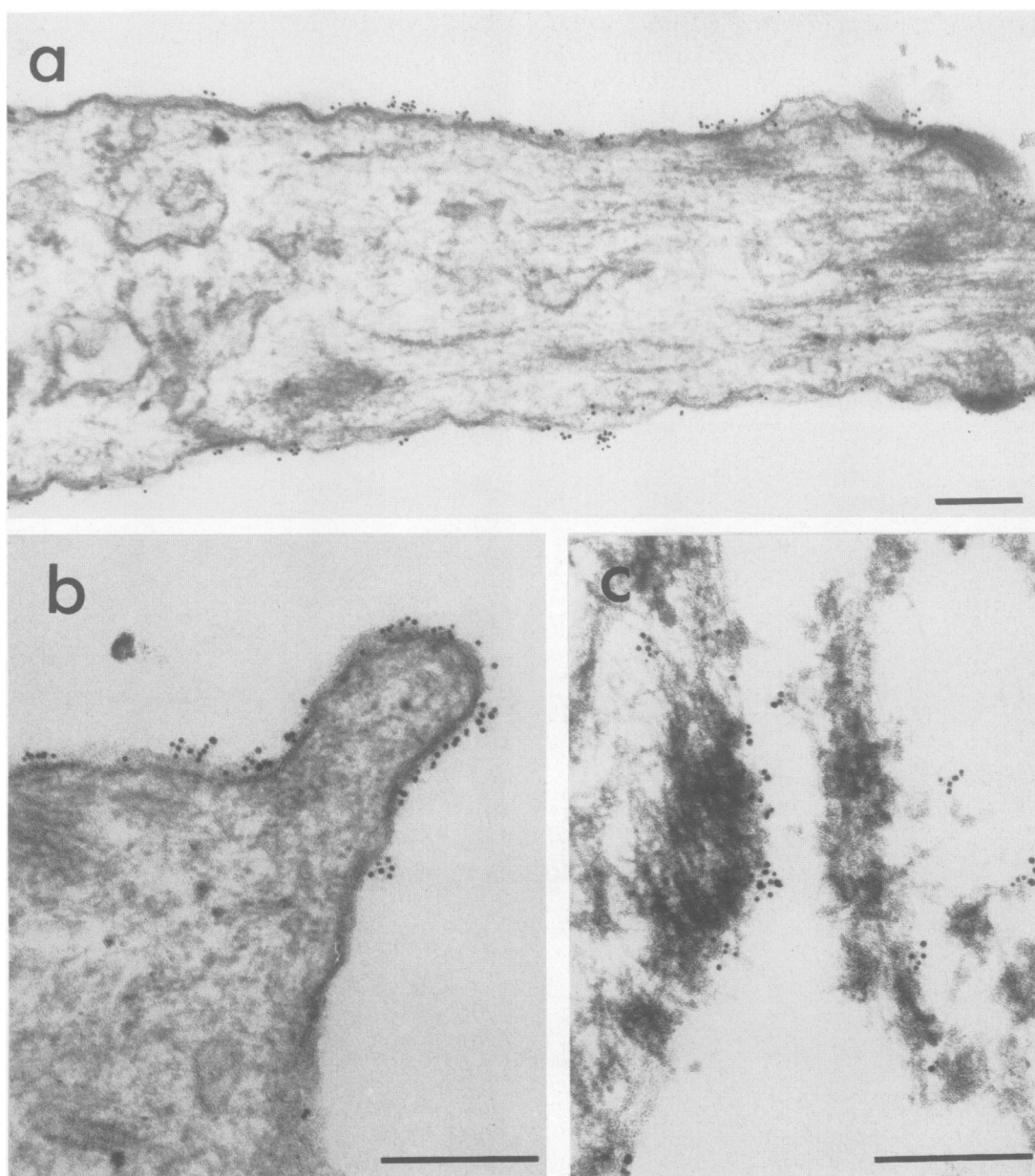
Our experimental procedures combine elements from the

two approaches mentioned above. (i) Partial purification of cardiac muscle ID. This fraction is enriched with adherens junctions. (ii) Selective extraction and isolation of membrane proteins from this fraction. (iii) Preparation of monoclonal antibodies to these components. (iv) Selection of monoclonal antibodies specific for adherens junctions of cardiac muscle and other organs. We used chicken cardiac muscle because it contains, in its intercalated discs, one of the typical and histologically defined adherens junctions, (*fascia adherens*). We have used a fraction which, in addition to ID, contained filamentous elements of the myofibrils, but did not contain non-junctional 'smooth' membranes because of the subsequent step of detergent extraction and partitioning. The latter procedure enabled an effective fractionation of the extracted proteins. Bordier (1981) and Coudrier *et al.* (1983) have suggested that cytoskeletal proteins and ECM elements are either insoluble or partition into the buffer phase while membrane proteins concentrate in the detergent phase. Therefore we used the detergent phase for immunization and later selected the hybridoma clones by differential radioimmunoassay (RIA) on the detergent phases of ID-rich and ID-poor fractions. The results of these RIA were immediately corroborated by immunofluorescent labelling of cardiac muscle and of culture myocytes.

As shown by indirect immunofluorescent labelling, ID-7.2.3 antibodies bind specifically to several intercellular junc-



**Fig. 7.** Immunofluorescent labelling of fixed (non-permeabilized) cultured chick cardiac cells (a–d) and chick lens cells (e–h) with ID-7.2.3 mAbs. Cells were either fixed and stained (a and e) or were pre-treated with EGTA prior to fixation. Time of incubation with EGTA was 3 min (f), 5 min (b and c) 10 min (d and g) or 30 min (h). Notice the exposure of the antigen due to the EGTA treatment and to the progressive separation of the two 'half-junctions'. The mirror-image nature of the distribution of the label in the two half-junctions is clearly apparent early after  $\text{Ca}^{2+}$  withdrawal (high power magnification in c). All pictures, except c were taken under the same magnification. The bars in c and h represent 10  $\mu\text{m}$ .



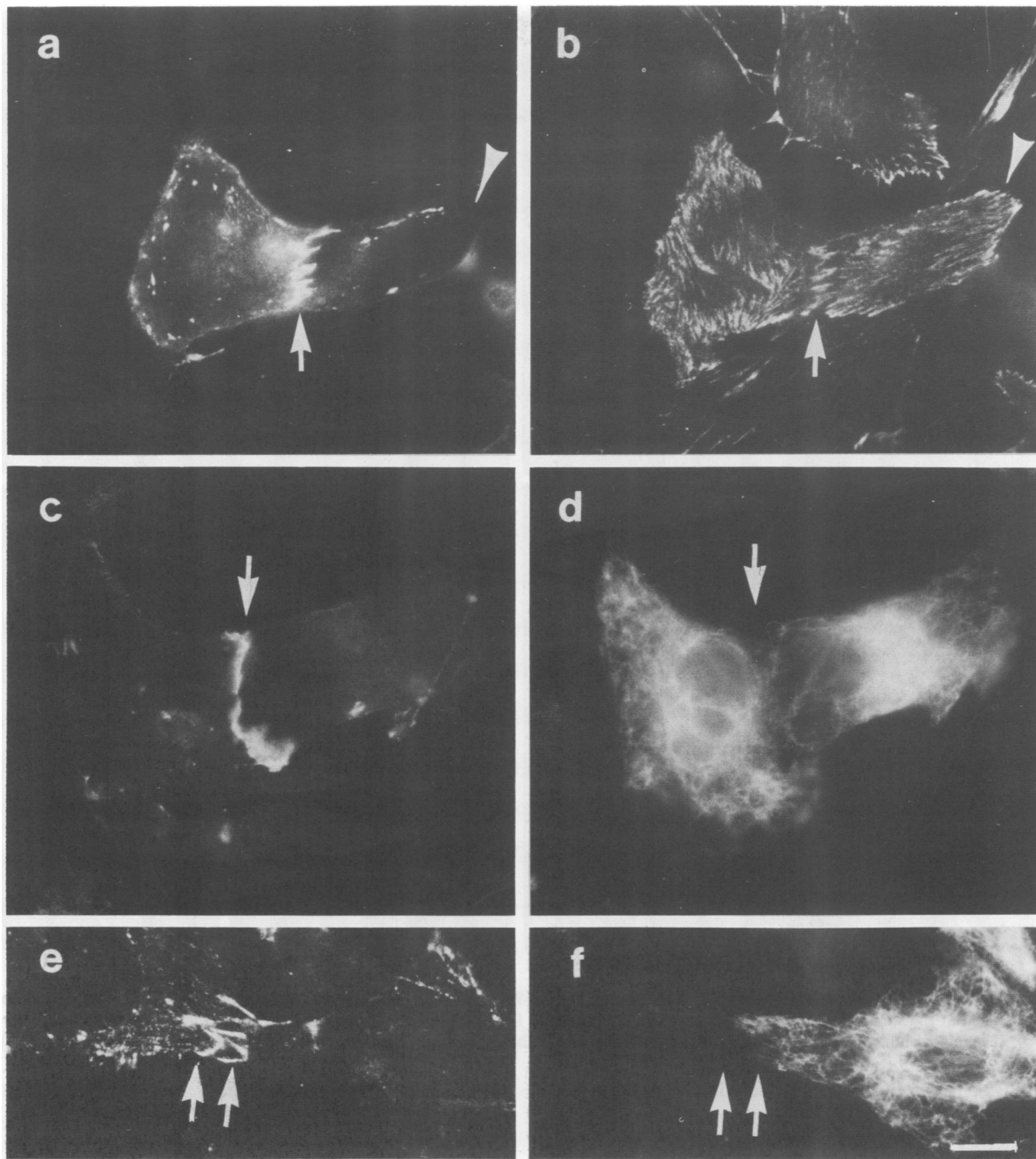
**Fig. 8.** Immunoelectron microscopic labelling of chicken cardiac cells with ID-7.2.3 mAbs. Cells were either treated with EGTA for 10 min, and fixed with glutaraldehyde (**a** and **b**), or permeabilized with Triton X-100 and then glutaraldehyde fixed. The labelling was indirect using mAb ID-7.2.3 and goat anti-mouse F(ab)<sub>2</sub> coupled to 5 nm gold. Bards indicate 0.2  $\mu$ m.

tions including elements in the intercalated discs of chick heart, contacts between lens fibers and intercellular contacts between cultured cells derived from chick heart, gizzard, lens and pigment epithelium. Immunoblotting indicated that ID-7.2.3 antibodies bind specifically to a 135-kd polypeptide and until additional information is available on its detailed molecular properties we refer to it as '135-kd protein'. The labelling was always exclusive to intercellular junctions while 'hemi adherens junctions' with the substrate or ECM, both in culture or in the intact tissues, were negative. This indicates that the molecular homology between the two types of contact, suggested by the presence of vinculin in both, is incomplete.

Several lines of evidence support our conclusion that the 135-kd protein is a component of intercellular adherens junctions. (i) In all cells and tissues studied the areas immunolabelled for the 135-kd protein were also positively labelled

for vinculin. According to our molecular definition (Geiger *et al.*, 1983) the presence of organized vinculin is a reliable marker for adherens junctions. Obviously, it would have been desirable to correlate the immunolabelling for the 135-kd protein directly with the morphology of the junction or with vinculin by electron microscopy. We are doing such experiments and preliminary results support the data obtained by immunofluorescent microscopy.

Other lines of evidence indicate that the ID-7.2.3 antibodies label specifically junctions of the adherens-type. (i) The labelling with ID-7.2.3 antibodies was often associated with belt-like structures (especially in cultures of lens cells or pigment epithelium). This pattern is typical of either tight or adherens-junctions in contrast to desmosomes and gap junctions. (ii) Desmosomes could also be excluded in light of the strong labelling of cells and tissues which contain no desmosomes or desmosome-like structures (lens cells,

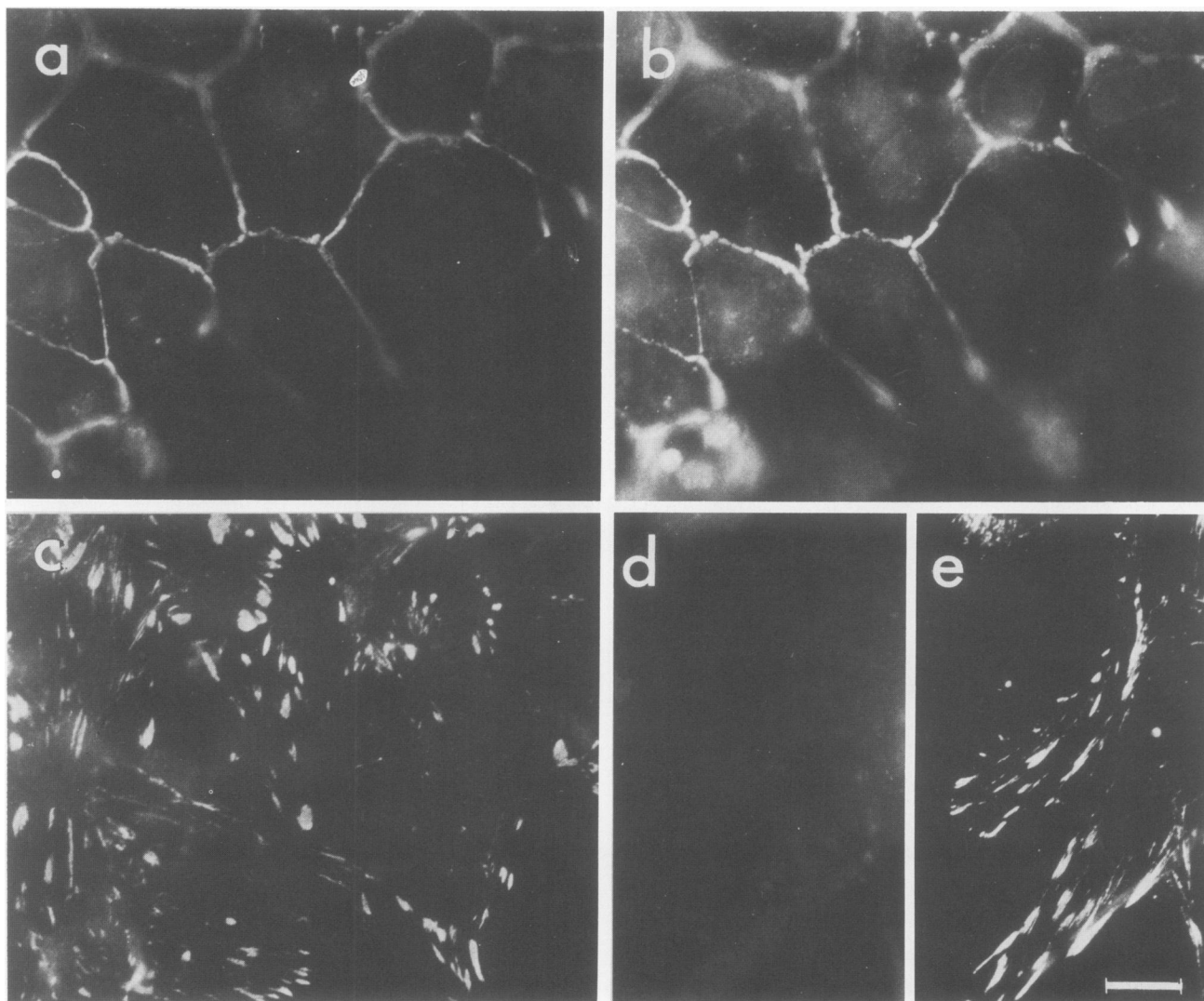


**Fig. 9.** Double immunolabelling of cultured chicken cardiac cells with ID-7.2.3 (**a**, **c** and **e**) and either rabbit anti-vinculin (**b**) or rabbit anti-desmin (**d** and **f**), respectively. The matched arrows in the pairs: **a** and **b**, **c** and **d**, and **e** and **f** point to the same locations. The matched arrowheads in **a** and **b** point to the absence of labelling with ID-7.2.3 from the vinculin-rich focal contacts. Bar indicates 10  $\mu\text{m}$ .

cultured fibroblasts, etc.). Moreover, double labelling of cultured cardiac cells showed that the 135-kd positive junctions were apparently independent of desmin which is attached to the *Maculae adherentes* in these cells (Kartenbeck *et al.*, 1983). (iii) The extensive labelling of cultured lens cells also excludes possible association with gap junctions. Conventional electron microscopic examination of these cells indicated that numerous and extended adherens junctions are formed between the cultured cells while gap junctions were seldom detected (not shown). Moreover, junction related to the 135-kd protein was highly dependent on the presence of  $\text{Ca}^{2+}$  ions this is typical of adherens — but not of gap junctions (Peracchia and Peracchia, 1980; Kartenbeck *et al.*, 1982). The intense staining of thin frozen sections of lens

tissue renders it unlikely that the 135-kd protein is associated with tight-junctions which were reported to be absent from this tissue (Benedetti *et al.*, 1976). On the basis of all this evidence we conclude that the 135-kd protein is a component related to the adherens-junction.

The immunofluorescent labelling of cultured cells raised an interesting point related to the topology of the 135-kd protein in or near the junction. The labelling of cardiac or lens cells required the exposure of the epitope either by detergent extraction or by short exposure to EGTA. Formaldehyde-fixed cells showed little labelling and viable cells were essentially negative. Since it was important to determine whether the antigen is membrane bound and facing the cell exterior we have run two control experiments. (i) We verified that

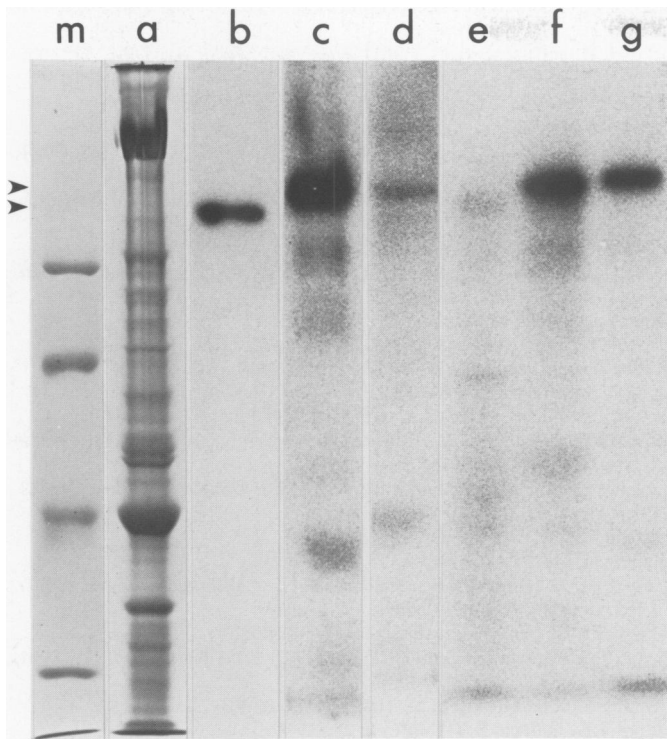


**Fig. 10.** Double immunofluorescent labelling of chick lens cells with mAbs ID-7.2.3 (a and d) and with rabbit anti-vinculin (b, c and e). The same group of cells was photographed at the subapical focal plane (a and b) or at the ventral focal plane (c). A single cell in d and e was photographed at the ventral focal plane. Notice the labelling of intercellular junctions with both antibodies and the absence of labelling with ID-7.2.3 of focal contacts. Bar indicates 10  $\mu$ m.

EGTA-treated cells remain intact and do not allow the penetration of antibodies into the cytoplasm. This was done using antibodies to defined cytoskeletal proteins on the same cells and in all cases no labelling was obtained (not shown). (ii) More direct evidence was provided by immunoelectron microscopic labelling of EGTA- or Triton-treated cells. In permeabilized chick heart cells the labelling was often associated with the cell boundaries in the neighbourhood of microfilament-associated, electron-dense plaques. The junction was not retained, however, under those conditions in an intact form and a better resolution was obtained with EGTA-treated cells. Since the treatment apparently disrupted the junction, the 135-kd protein in these cells was not confined to specific areas and the labelling was distributed over the surfaces of many cells. The gold particles were closely and exclusively associated with the membrane suggesting that the epitope was an integral component of the membrane and not adsorbed on its surface (staining of similar cells for fibronectin, for example, resulted in extensive labelling of the substrate and of intercellular matrices (not shown). We should emphasize that the EGTA experiments indicated that the 135-kd protein may diffuse laterally in the plane of the

membrane unless its mobility is restricted by 'vertical' interactions with the neighbouring cell on the exterior or with the cytoskeleton.

Little information is available on the molecular properties of the 135-kd protein. The mol. wt. was estimated from immunoblotting analysis of electrophoretic gels and its presence in a variety of tissues was examined by the same technique. In the last few years several investigators have reported the isolation of adhesion- (or aggregation-) related molecules with similar mol. wts. The relationships between the 135-kd protein described here and 120–150 kd polypeptides described by others (Takeichi, 1977; Rutishauser *et al.*, 1978a, 1978b; Urushihara and Takeichi, 1980; Knudsen *et al.*, 1981; Imhof *et al.*, 1983) are still not clear. The unique property of the 135-kd protein is its specific localization in a defined microfilament-bound intercellular junction. To the best of our knowledge this is the first membrane component of the intercellular adherens junction to be identified. Other adhesion-related molecules were identified by functional assays (antibody-induced inhibition of attachment or aggregation) but they were not localized at a specific intercellular junction. Recently, Oesch and Birchmeier (1982)



**Fig. 11.** Immunoblotting analysis of different chicken tissues (separated on 8% polyacrylamide gel) with mAb against vinculin (b) or with ID-7.2.3 (c–g). The tissues tested were chicken heart (b and c), skeletal muscle (d), liver (e), brain (f) and eye lens (g). a is a Coomassie blue-stained gel of chicken cardiac muscle. m, markers, including phosphorylase b, BSA, ovalbumin, carbonic anhydrase. The arrowheads mark the location of the 135-kd protein (upper) and vinculin (lower).

described inhibition of cell adhesion by a monoclonal antibody directed against a 60-kd protein. The labelling with this antibody was reported to be specific for the surface of cell-to-substrate (but not cell-to-cell) contacts. This is in contrast to our 135-kd protein which is located in intercellular contacts only. Further characterization of the 135-kd protein will hopefully shed light on the molecular interactions involved in intracellular recognition and junction biogenesis.

## Materials and methods

### Intercalated disc (ID)-rich fraction

ID-rich membranes were isolated from chick cardiac muscle by a modification (see Figure 1) of the method of Colaco and Evans (1981, 1982). 0.5 mM phenylmethylene sulfonyl fluoride (PMSF) was added routinely to the extraction solutions just before use. Samples from all the stages of purification were examined by electron microscopy to select for the ID-rich fractions. Additional details are given in the Results section.

### Extraction with Triton X-114 and detergent partitioning

Triton X-114 (Sigma, USA) was precondensed three times in 10 mM Tris-HCl buffer, pH 7.4 according to Bordier (1981). Concentration of the detergent was determined spectroscopically from the absorbance at 275 nm and stock solutions of 10–20% stored at 4°C. Suspensions of P45–52 or P52–54 (containing ~1 mg protein/ml) were extracted in 10 mM Tris HCl, 150 mM NaCl, 1% Triton X-114 pH 7.4 at 0°C. The extract was cleared by centrifugation in the cold (12 000 g, 5 min) and loaded on 1.5 volumes 10 mM Tris-HCl buffer, 0.6% sucrose, 150 mM NaCl, 0.06% Triton X-114 pH 7.4 and incubated for 10 min at 37°C. This temperature is above the cloud point of Triton X-114 and thus phase separation was obtained as described by Bordier (1981). In principle, four fractions were obtained after this fractionation: (1) total detergent extract; (2) detergent-insoluble residue; (3) detergent (lower) phase obtained after detergent partitioning at 37°C; (4) buffer (upper) phase obtained after detergent partitioning at 37°C.

### Gel electrophoresis

Polyacrylamide gel electrophoresis was performed in Laemmli buffer system (Laemmli, 1970) on slab 8% or on 6–15% polyacrylamide gels. Gels were usually stained with Coomassie blue and mannose-containing bands identified by the [<sup>125</sup>I]Con A overlay technique (Burrage, 1978). Immunoblotting was performed according to Towbin *et al.* (1979). The separated protein bands were electroblotted in the cold onto nitrocellulose sheets. The nitrocellulose was incubated with 3% bovine serum albumin (BSA) in 10 mM Tris-HCl buffer, 150 mM NaCl, pH 7.4, and then incubated with antibody solutions for 3 h. The sheet was then rinsed, in PBS containing 1% BSA, incubated with <sup>125</sup>I-labelled goat anti-mouse F(ab')<sub>2</sub>, rinsed again and analysed by autoradiography. For the [<sup>125</sup>I]Con A overlay technique (Burrage, 1978), we have used Con A solution containing 10<sup>6</sup> c.p.m./ml and incubated it with the Coomassie blue-stained gels for 5 h. The gels were then washed for about 48 h, dried and autoradiographed.

### Immunochemical reagents

The various antigens (0.6 mg/ml) were emulsified in complete Freund's adjuvant and injected i.p. and into the footpads of 3 month old female (BALB/c x DBA/2)F1 mice (CD/2). Three weeks later each mouse was boosted by the same dose of antigen injected i.p. and s.c. in incomplete Freund's adjuvant. Positively reacting mice (determined by RIA, see below) were injected again, 3 weeks after the challenge injection and 3 days later their spleens were removed and used for fusion. The preparation of hybridomas and their maintenance were carried out according to Eshhar *et al.* (1979). The myeloma used for fusion was NSO (Galfre and Milstein, 1981), which were kindly supplied by Dr. Z.Eshhar from our Department. Selected reacting hybridoma lines were cloned in soft agar and the clones propagated either in culture or in ascites form in Prystane treated CD/2 mice.

Other antibodies used in this study were as follows. Anti-vinculin was prepared in rabbits, guinea pigs or mice (monoclonal), and affinity purified before use (Geiger, 1979; Geiger *et al.*, 1980). Pure antibodies to chicken gizzard desmin were prepared and purified as described previously (Geiger and Singer, 1980). As secondary antibody reagents we have used goat anti-rabbit Ig and goat anti-mouse F(ab')<sub>2</sub>, both affinity purified. These antibodies were coupled to rhodamine-lissamine sulfonyl chloride or to dichlorotriazinyl amino fluorescein as previously described (Brandtzaeg, 1973; Geiger and Singer, 1979; Avnur and Geiger, 1981).

### Radioimmunoassay

Detergent-solubilized proteins (60 µg/ml) were adsorbed onto polylysine-coated wells of microtiter plates (Cook Laboratories, USA). After 2 h at 25°C the plates were rinsed with PBS containing 1% BSA. Hybridomas supernatants were then applied to the wells and incubated for 2 h. The wells were rinsed, again with PBS-BSA, incubated with [<sup>125</sup>I]goat anti-mouse F(ab')<sub>2</sub> (~7 x 10<sup>4</sup> c.p.m./well), rinsed and individual wells counted in a gamma scintillation counter.

### Immunohistochemical labelling

Thin frozen sections (0.5–1 µm thick) were prepared according to Tokuyasu (1980; see also Griffiths *et al.*, 1983), in the Sorvall MT2B equipped with a cryoattachment. The sections were retrieved with a platinum loop in 2.3 M sucrose droplet and stained as described earlier (Geiger *et al.*, 1979). Cultured cells used here for immunofluorescent labelling were prepared from three alternative sources. (i) Gizzard cells were prepared from 8–10 day old chick embryos. (ii) Lens cell cultures were prepared from trypsin-suspended chick lens cells following incubation for 3–7 days in culture. The cells exhibited an epithelioid morphology and formed dense sheets. We have used cells from either primary cultures or up to the fifth passage. (iii) Primary heart myocytes in culture were enriched by plating of trypsin-suspended cells of embryonic chick hearts (8–10 days) on 10 cm Falcon dishes for 1 h. Most of the fibroblasts adhered to the dish while most of the myocytes remained in suspension. The supernatant was then transferred to other tissue culture plates (Falcon) or to coverslips and incubated for additional 2 days. The latter culture was highly enriched (>50%) with contracting heart myocytes, which were also desmin positive.

Cells were fixed in two alternative ways: (i) cells were permeabilized by short (2 min) exposure to 0.5% Triton X-100 in 50 mM morpholinoethane sulfonate (MES) buffer, 3 mM EGTA, 5 mM MgCl<sub>2</sub> pH 6.0 followed by fixation with 3% paraformaldehyde or (ii) cells were fixed as above without permeabilization. Fluorescence microscopy was performed with a Zeiss photomicroscope III.

### Electron- and immunoelectron-microscopy

Various samples including intact tissues, membrane suspensions or cultured cells on Falcon dishes were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, 5 mM CaCl<sub>2</sub> pH 7.2. The samples were post-fixed with 1% osmium tetroxide, washed and stained *en bloc* with 1% uranyl acetate. After dehydra-

tion the samples were embedded in Poly Bed 812 (Polysciences, USA), and sections cut with LKB microtome. Flat embedded cells in monolayer cultures were re-embedded in epon and sectioned as above.

Immunoelectron microscopic labelling was carried out with either fixed cells or with detergent-permeabilized cells. The cell monolayers were incubated with ID-7.2.3 antibodies (1:20 dilution of ascites fluid or hybridoma culture supernatant, used without dilution) for 30 min, washed and then labelled with affinity-purified goat anti-mouse F(ab')<sub>2</sub> coupled to 5 nm gold (coupling of the gold to our antibodies was performed by Drs. J.De May and M.Moremans from Janssen Pharmaceutica, Beerse, Belgium). After labelling the cultures were refixed in 2% glutaraldehyde and processed further for thin sectioning as above. Sections were stained with uranyl acetate and lead citrate and examined in the Philips EM 300 electron microscope.

## Acknowledgements

We acknowledge with gratitude the excellent assistance of Ms. T.Volberg. This study was supported by a grant from the Muscular Dystrophy Association.

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Received on 22 June 1984