Proc. Natl. Acad. Sci. USA
Vol. 78, No. 12, pp. 7619–7623, December 1981
Cell Biology

Ultrastructure of chicken cardiac muscle as studied by double immunolabeling in electron microscopy

(vinculin/α-actinin/intercalated disc membrane/cryo-ultramicrotomy)

K. T. TOKUYASU, ANNE H. DUTTON, BENJAMIN GEIGER*, AND S. J. SINGER

Department of Biology, University of California at San Diego, La Jolla, California 92093

Contributed by S. J. Singer, September 9, 1981

ABSTRACT **The ultrastructural localization of α-actinin and vinculin in chicken cardiac muscle was studied by double indirect immunoelectron microscopy, using ferritin and iron–dextran (Imposil) as the electron-dense markers conjugated to the secondary antibodies, on ultrathin frozen sections of fixed tissue. Fixation and immunolabeling procedures were developed that permitted maximal retention of the two proteins at their natural sites as well as their adequate labeling. α-Actinin was found both on the Z-bands, as expected, and near the fascia adherens of the intercalated discs, whereas vinculin was confined to the latter sites. At the fascia adherens, the double labeling results clearly showed that vinculin was situated closer to the membrane than was α-actinin. These results, coupled with earlier observations, suggest that vinculin may participate in the linkage of actin-containing microfilament bundles to membranes in a variety of cell types.**

The linkages of microfilaments to membranes in a variety of cells play important roles in cell motility, cell adhesion, cell–cell interactions, and contractility. The precise molecular architecture of these linkages is not yet understood. To study the specific molecular components present at sites of microfilament–membrane attachments, and to obtain information about their arrangements at these sites, we have been using techniques developed in our laboratory for the immunolabeling of specific proteins on ultrathin frozen sections of appropriately fixed tissues (1–3). α-Actinin (4) and vinculin (5, 6) are two proteins that have been proposed to participate in microfilament–membrane linkages. We have therefore investigated the immunolabeling of these two proteins in different tissues. Initially, these proteins were labeled individually on separate samples of the same tissues (6, 7). More recently, we have developed general methods for the double immunolabeling of any two antigens on the same electron microscopic specimen (8). For this purpose, two distinguishable electron-dense labels covalently bound to antibody molecules are utilized, ferritin and iron–dextran complexes (Imposil). The iron oxyhydroxide cores of the Imposil particles, which are the objects seen in transmission electron microscopy, are anisometric and readily distinguished from the isometric cores of ferritin molecules.

In this paper and in ref. 9 we report the application of this double immunolabeling technique on ultrathin frozen sections of ultrastructurally intact tissues. Here the proteins vinculin and α-actinin have been studied by double immunolabeling of chicken heart muscle. With appropriate fixation conditions, we show that vinculin and α-actinin are both located close to the intercalated disc membrane, specifically at the fascia adherens junctions, with vinculin situated closer to the membrane at these sites than is α-actinin. Whereas α-actinin is also found at the Z-lines in the myofibrils (10), vinculin is confined to the intercalated disc (6). These results confirm that the fascia adherens and the Z-line structures, although both are sites of attachment of actin microfilaments, are nevertheless different structural entities and suggest that vinculin may participate in the linkages of the microfilaments to the membrane of the fascia adherens more directly than does α-actinin.

MATERIALS AND METHODS

The affinity-purified rabbit antibodies to chicken gizzard α-actinin and guinea pig antibodies to chicken gizzard vinculin that were used as the primary antibodies, the affinity-purified and cross-absorbed goat antibodies to rabbit IgG and to guinea pig IgG, the conjugation of the goat antibodies to either ferritin or Imposil, and the procedures for ultrathin frozen sectioning and immunolabeling, unless otherwise specified, were all as described in detail elsewhere (9). The ventricular papillary muscle of chicken heart was dissected to blocks of 1-mm dimensions or smaller in one of two fixative solutions, each of which was the first stage of a different fixation procedure: (*i*) 3% (wt/vol) paraformaldehyde plus 20 mM ethylacetimidate in phosphate-buffered saline (P_i/NaCl) containing 2 mM $CaCl_2$ for 2–10 min, or (*ii*) 8% (wt/vol) paraformaldehyde in P_i/NaCl plus 2 mM $CaCl_2$ for 1 hr. In the first case, the first stage was followed by treatment with 3% paraformaldehyde plus 0.1% glutaraldehyde. We refer to this two-stage treatment as the *mild fixation procedure*. In the second case, the first stage was followed by treatment with a mixture of 8% paraformaldehyde plus 20 mM ethylacetimidate in P_i/NaCl containing 2 mM $CaCl_2$ for 5 min, and finally a mixture of 4% (wt/vol) paraformaldehyde plus 4% (wt/vol) glutaraldehyde in P_i/NaCl containing 2 mM $CaCl_2$ for 1 hr. We refer to this three-stage treatment as the *strong fixation procedure*. These fixatives included $CaCl_2$ in order to avoid separation of the muscle cells from one another. The inclusion of $CaCl_2$ produced a large nonspecific background staining with Imposil conjugates, which was overcome by including 1% dextran (M_r 10,000) in the solution of 2% gelatin used to condition the sections just prior to immunolabeling.

Double indirect immunolabeling of the ultrathin frozen sections was carried out as follows (9). The primary antibodies (rabbit antibodies to α-actinin and guinea pig antibodies to vinculin) were mixed and applied together to the section; after thorough rinsing, the two cross-adsorbed secondary antibody conjugates (ferritin-conjugated goat antibodies to rabbit IgG and Imposil-conjugated goat antibodies to guinea pig IgG) were mixed and applied together to the section. This procedure results in the labeling of α-actinin with ferritin and vinculin with Imposil particles. After the immunolabeling, the sections were positively stained by the adsorption method (11) involving the following

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: P_i/NaCl, phosphate-buffered saline.

* Present address: Department of Chemical Immunology, Weizmann Institute, Rehovot, Israel.

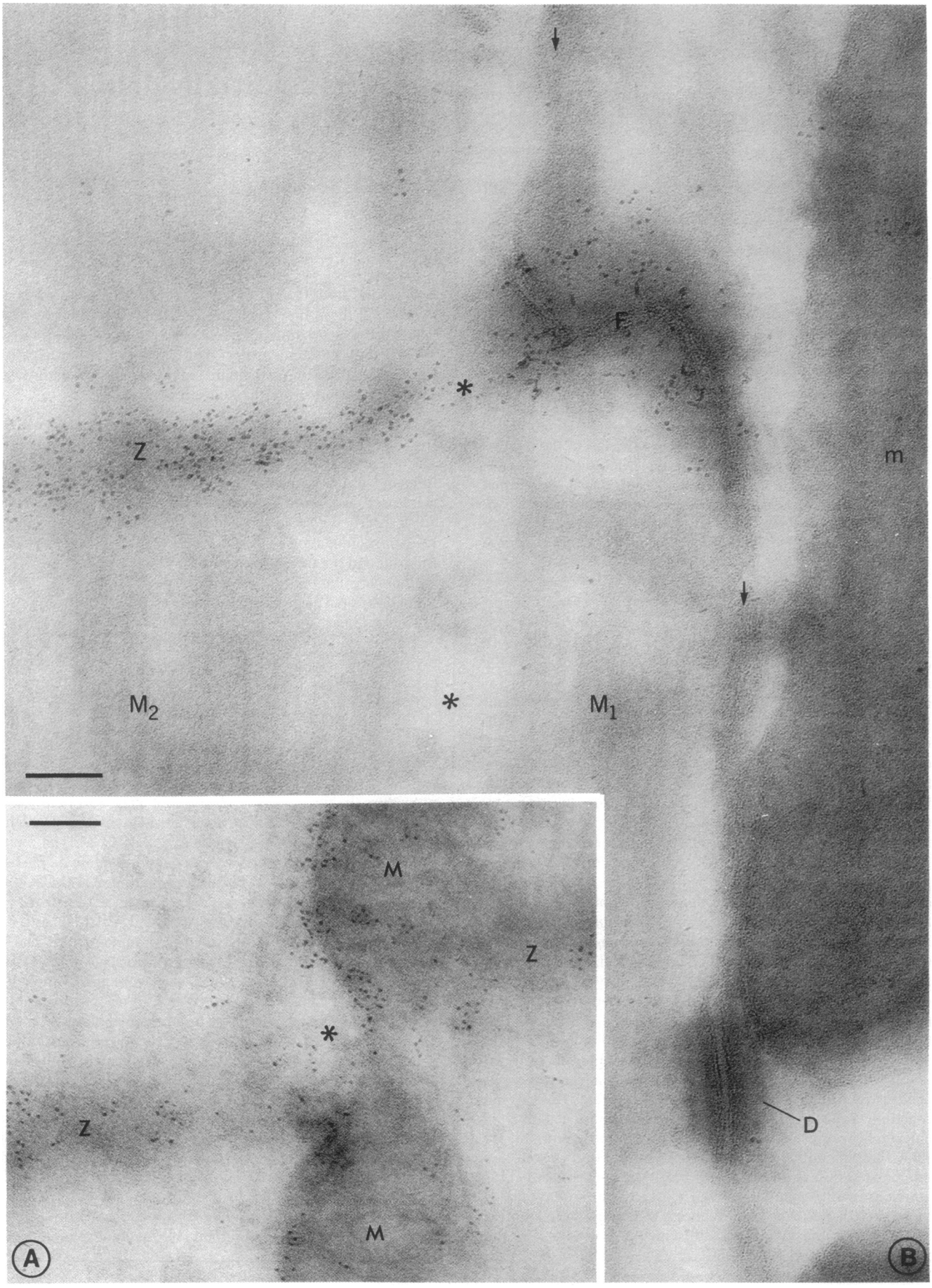

FIG. 1. *(Legend appears at the bottom of the next page.)*

sequence of steps: (*i*) 2% neutral uranyl acetate for 10 min, (*ii*) thorough washing with H_2O, and (*iii*) 0.02% acidic uranyl acetate/1% methylcellulose/1% Carbowax (polyethylene glycol; M_r 1540) for 10 min, followed by drying (embedding) in a thin layer of this last mixture without further washing. Sections were examined in a Philips model 300 electron microscope operated at 60 kV.

RESULTS

Vinculin and α-actinin are a pair of proteins that provide a stringent test of the generality of the method of double immunoelectron microscopic labeling. They are particularly difficult to immunolabel on the same fixed ultrathin frozen section. This is due on the one hand to the total inactivation of vinculin by the presence of glutaraldehyde in the primary fixative (9) and on the other hand to the tendency of α-actinin to redistribute artifactually in the specimen upon too mild a fixation (7). The two-stage mild fixation procedure described in *Materials and Methods* has been used successfully with other systems (2), but when used with the chicken heart muscle, appeared to allow an artifactual redistribution of α-actinin in the sections (Fig. 1*A*). From the extent of labeling, a significant quantity of α-actinin was apparently dissociated from the Z-line (compare *A* and *B* in Fig. 1) and relocated to other areas such as over mitochondria and intermyofibrillar spaces (Fig. 1*A*) that are spurious locations for that protein.

In order to fix α-actinin more firmly to its sites without inactivating vinculin, and also to better preserve the ultrastructure of the muscle, we then resorted to the three-stage strong fixation procedure described in *Materials and Methods*. Under these conditions, the ferritin labeling for α-actinin was very dense on the Z-band (Figs. 1*B* and 2*B*) and near the fascia adherens (Figs. 1*B* and 2*A*), while on the same specimens, Imposil labeling for vinculin was exclusively at the fascia adherens. In such fields the Imposil particles were as a whole clearly situated closer to the membrane of the fascia adherens than were the ferritin particles, suggesting that vinculin is localized nearer the membrane than is α-actinin at these sites. Neither vinculin nor α-actinin labeling was localized on or near the desmosomes, whether situated on the lateral plasma membranes (Fig. 1*B*) or adjacent to the fascia adherens at the intercalated disc (Fig. 2*A*).

In experiments not shown here, the secondary labeling reagents were reversed; that is, ferritin-conjugated goat antibodies to guinea pig IgG and Imposil-conjugated goat antibodies to rabbit IgG were used with the same primary antibodies as previously employed. Under these circumstances, on strongly fixed specimens, the Imposil labeling for α-actinin was much less intense than was the ferritin labeling for α-actinin (Figs. 1*B* and 2 *B* and *C*) that was obtained with the first set of secondary reagents. This suggests that secondary ferritin conjugates had greater accessibility than did the somewhat larger Imposil conjugates to the primary antibodies attached to α-actinin in these fixed sections.

A typical control experiment is shown in Fig. 2*C*. In this instance, normal rabbit IgG was substituted for the primary rabbit antibodies, and it was followed by the ferritin-conjugated goat antibodies to rabbit IgG. No significant labeling of any area of the specimen was observed (the fascia adherens is shown) in this or similar controls.

DISCUSSION

In this paper, we report the application of a technique of double immunoelectron microscopic labeling of two antigens on the same ultrathin frozen sections of a tissue specimen, namely the labeling of two proteins, vinculin and α-actinin, in chicken cardiac muscle. A paper appearing elsewhere (9) describes the technology in detail and includes other double immunoelectron microscopic experiments with combinations of the proteins vinculin, α-actinin, and tropomyosin on ultrathin frozen sections of chicken gizzard smooth muscle. In chicken cardiac muscle, we confirmed earlier findings that α-actinin is present on the Z-line of striated muscle sarcomeres (10) and vinculin is present at the fascia adherens of the intercalated disc membrane (6). Our finding that α-actinin is also present at the fascia adherens (Figs. 1*B* and 2*A*) is a new result; to the best of our knowledge there are no previously published micrographs demonstrating this point. Most interesting, however, is the clear segregation of the labeling for vinculin and α-actinin at the fascia adherens (Figs. 1*B* and 2*A*) with the vinculin situated closer to the membrane at these sites than α-actinin. The power of the double immunolabeling method in ultrastructural analysis is revealed by these results.

These findings have strong parallels with those that we have obtained in related experiments with other tissues. In chicken gizzard smooth muscle cells, both the membrane-associated dense plaques and the cytoplasmic dense bodies were immunolabeled for α-actinin (9), but only the former dense plaques were labeled for vinculin. At the dense plaques, double immunolabeling for vinculin and α-actinin showed that the vinculin labeling was situated closer to the cell membrane than was α-actinin labeling. In the brush border of chicken intestinal epithelium (7, 9) α-actinin was found in several locations, but vinculin labeling was confined to the region close to the zonula adherens. At the zonula adherens, vinculin was situated closer to the membrane than was α-actinin. Similar double immunoelectron microscopic results have been obtained at the focal adhesion plaques, where cultured fibroblasts adhere to their substrata (unpublished data). The four sites—the fascia adherens of the intercalated disc of cardiac striated muscle, the membrane-associated dense plaques of smooth muscle, the zonula adherens of the junctional complex of epithelial cells, and the membrane-associated plaques at the focal adhesions of cultured fibroblasts—all sites at which microfilament bundles terminate at the plasma membrane—appear to have a remarkably similar composition and ultrastructure despite the marked differences in cell types and structures. At each of these sites vinculin is situated close to the membrane and α-actinin is somewhat further away. It has been proposed, therefore, that vinculin may participate in the linkage of the microfilaments to the membranes at these sites (6, 9), perhaps as a peripheral protein of the membrane. Vinculin is clearly a better candidate for this function than is α-actinin.

FIG. 1 (*on preceding page*). All figures are electron micrographs of longitudinal frozen sections of chicken cardiac muscle cells that were double immunolabeled with ferritin labels for α-actinin and Imposil labels for vinculin, except Fig. 2*B*, which shows a control section. Scales in the micrographs indicate 0.1 μm. (*A*) Part of a section of a specimen fixed by the two-stage mild procedure. Ferritin particles that represent α-actinin are found not only on the Z-bands (Z) but also on the sections of mitochondria (M), in the intermyofibrillar space (asterisk), and in the myofibrillar areas. No Imposil particles representing vinculin are seen in this field. (×130,000.) (*B*) Section of a strongly fixed specimen. The lateral cell border (arrows) forms a narrow step consisting of a fascia adherens (F), across the width of a myofibril (M_1) that is in register with a Z-band (Z) of an adjacent myofibril (M_2) (asterisks indicate the inter-M_1–M_2 space). Numerous ferritin particles representing α-actinin are seen on the Z-band and along the fascia adherens. Imposil particles representing vinculin are as a whole localized closer to the plasma membrane of the fascia adherens than are ferritin particles. A desmosome (D) is observed on the lateral cell border; neither Imposil nor ferritin particles are recognized near it or on its profile. m, Mitochondrion. (×140,000.)

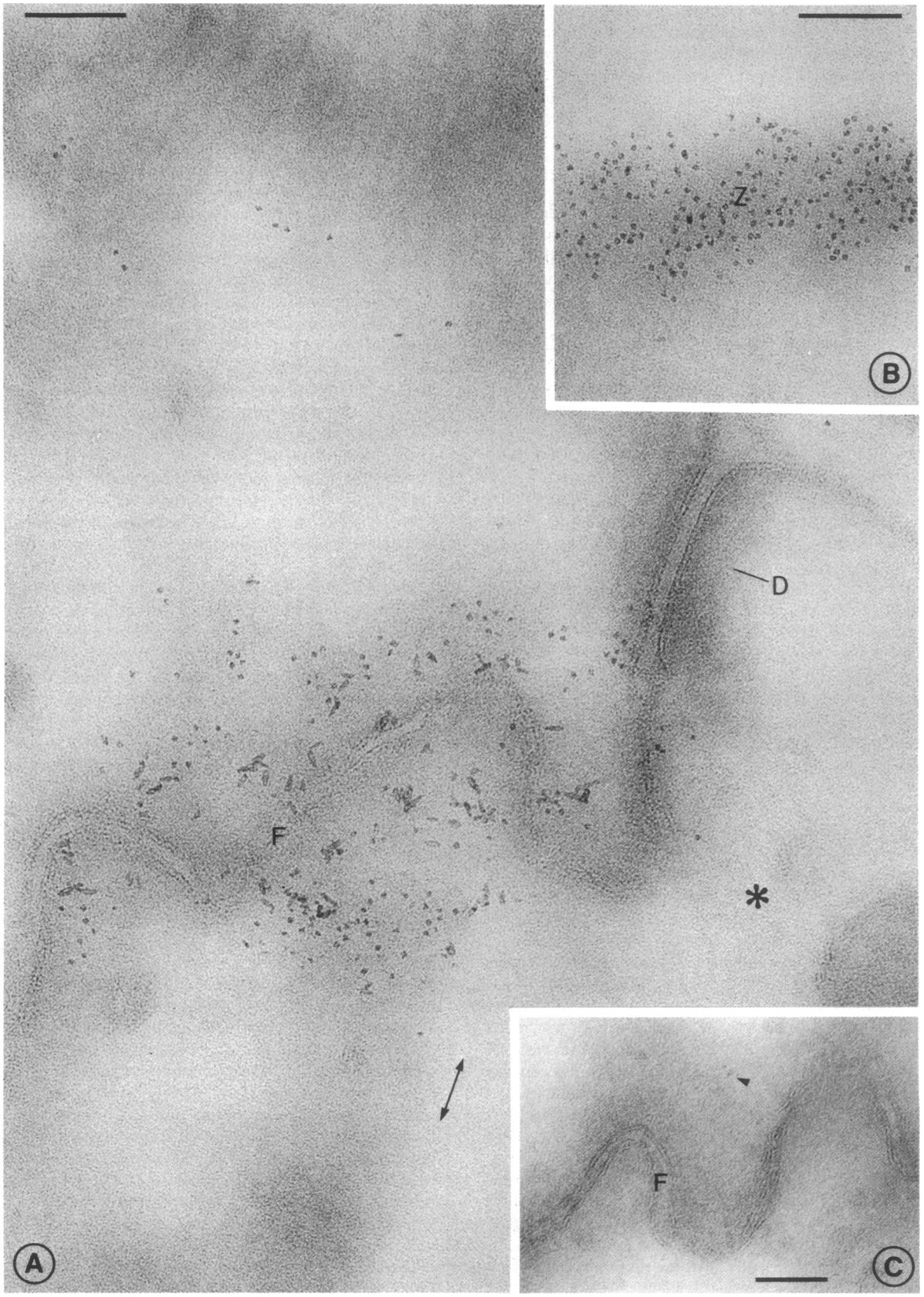

FIG. 2. (*A* and *B*) Adjacent parts of a section of a strongly fixed specimen. In *A*, both ferritin particles representing α-actinin and Imposil particles representing vinculin are recognized on the fascia adherens (F) of the intercalated disc (double-headed arrow indicates the direction and the lateral boundary of the myofibril, and asterisk indicates the space between the myofibril and the lateral plasma membrane). Here, Imposil particles as a whole are obviously more closely associated with the plasma membrane of the fascia adherens than are ferritin particles. In *B*, only ferritin particles are observed on the Z-band. No significant labeling of either α-actinin or vinculin is seen on the desmosome (D) that is located next to the fascia adherens within the intercalated disc. m, Mitochondrion. (*A* and *B* both ×170,000.) (*C*) Control section of the same specimen as shown in *A* and *B* that was treated first with normal rabbit IgG and then with ferritin-conjugated goat antibodies to rabbit IgG. The background level is so low that only a careful inspection reveals a few ferritin particles (arrowhead) near the fascia adherens (F). (×120,000.)

In many discussions of muscle cellular physiology in the past, the Z-bands and the intercalated discs of cardiac striated muscle and the cytoplasmic dense bodies and membrane-associated dense plaques of smooth muscle have all been considered as analogous structures. The present and other results (6, 9), however, clearly discriminate among them. The Z-bands and cytoplasmic dense bodies are intracellular nonmembranous structures that are analogous to one another in their association with α-actinin, and the intercalated discs and dense plaques are alike in being membrane-associated and containing vinculin, but the two pairs of structures clearly differ from each other.

The technology of double immunoelectron microscopic labeling experiments having only recently been introduced (8), we have encountered some problems in the present study whose solutions should help to make the technology more generally applicable. For example, while α-actinin is apparently antigenically quite stable to a variety of fixation treatments, many of these do not adequately fix the protein to its natural sites in a variety of tissues that we have examined (7, 9). Stronger fixation conditions such as the three-stage procedure used in this paper overcame this problem in cardiac muscle specimens. These conditions also allowed the retention of the antigenicity of vinculin, a protein that is sensitive to the direct action of glutaraldehyde (9). It was also found that, under these fixation conditions, immunolabeling for α-actinin with a secondary ferritin-antibody conjugate was much more effective than with a secondary Imposil-antibody conjugate. The strong fixation conditions may have so extensively crosslinked the surface of the section that it was much less penetrable by the somewhat larger Imposil conjugate particle than by the smaller ferritin one. With attention given to these factors, it was possible to carry out satisfactory specific double immunolabeling for the two proteins on the same sections of cardiac muscle tissue. These methods should have general applicability to many other systems.

We are happy to acknowledge the excellent technical assistance of Mrs. Margie Adams and Mr. J. Michael McCaffery. B.G. was a Chaim Weizmann Foundation Postdoctoral Fellow, 1977–1979. S.J.S. is an American Cancer Society Research Professor. These studies were supported by U.S. Public Health Service Grant GM-15971.

1. Tokuyasu, K. T. (1973) *J. Cell Biol.* **57**, 551–565.
2. Tokuyasu, K. T. & Singer, S. J. (1976) *J. Cell Biol.* **71**, 894–906.
3. Singer, S. J., Tokuyasu, K. T., Dutton, A. H. & Chen, W.-T. (1981) in *Electron Microscopy in Biology*, ed. Griffith, J. D. (Wiley, New York), Vol. 2, in press.
4. Mooseker, M. S. & Tilney, L. G. (1975) *J. Cell Biol.* **67**, 725–743.
5. Geiger, B. (1979) *Cell* **18**, 193–205.
6. Geiger, B., Tokuyasu, K. T., Dutton, A. H. & Singer, S. J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4127–4131.
7. Geiger, B., Tokuyasu, K. T. & Singer, S. J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 2833–2837.
8. Dutton, A. H., Tokuyasu, K. T. & Singer, S. J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3392–3396.
9. Geiger, B., Dutton, A. H., Tokuyasu, K. T. & Singer, S. J. (1981) *J. Cell Biol.*, in press.
10. Masaki, T., Endo, M. & Ebashi, S. (1967) *J. Biochem.* **62**, 630–632.
11. Tokuyasu, K. T. (1980) in *Proceedings, Electron Microscopy Society of America, 38th Meeting*, ed. Bailey, G. W. (Claitor, Baton Rouge, LA), pp. 760–763.