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Vinculin, an intracellular protein localized at specialized sites where microfilament bundles terminate at cell membranes

(intestinal epithelial brush border/smooth muscle/cardiac muscle/immunoelectron microscopy/cryoultramicrotomy)

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ABSTRACT **An intracellular protein of 130,000 molecular weight was recently isolated in this laboratory from chicken gizzard smooth muscle. By immunofluorescence observations of cultured chicken fibroblasts, it was shown to be concentrated on the ventral surfaces of the cells where they formed focal adhesions to the substratum [Geiger, B. (1979) *Cell* 18, 193–205]. Focal adhesions are sites where, inside the fibroblast, microfilament bundles are known to terminate at the cell membrane. The suggestion was made that this new protein (herein named "vinculin") might be involved in the linkage of the termini of microfilament bundles to membranes in various cell types. To explore this possibility, in the present study we examined several chicken tissues, including intestinal epithelium, gizzard smooth muscle, and cardiac striated muscle, by immunoelectron microscopic labeling for vinculin on ultrathin frozen sections of the specimens. In each case, the immunolabeling for vinculin was concentrated close to membrane sites where microfilament bundles terminate: at the zonula adherens in the junctional complex of the brush border of epithelial cells; at the membrane-associated dense plaques of smooth muscle cells; and at the fascia adherens of the intercalated disk membranes of cardiac muscle cells. These results suggest therefore that vinculin may participate in the anchoring of microfilament bundles to specific membrane sites in various cells.**

Actin is a ubiquitous protein in the cytoplasm of eukaryotic cells. In the form of F-actin, it is the major component of the 50- to 70-Å-diameter filaments (microfilaments) inside many types of cells. Microfilaments together with myosin and associated structural and regulatory proteins provide the molecular machinery for much of the contractile activity of nonmuscle as well as muscle cells (1, 2). An important factor in this contractile activity is the attachment of the termini of bundles of microfilaments to specialized regions of the plasma membrane of a cell. This attachment provides one type of anchor against which the contractile machinery can exert tension. In different types of cells, microfilaments exist in quite different states of organization and, correspondingly, their regions of attachment to membranes appear quite different structurally in transmission electron microscopy. In cardiac striated muscle cells, for example, the microfilaments form part of a highly organized sarcomere structure and terminate at specialized sites of the intercalated disk membranes called fascia adherens (3). In the brush border of intestinal epithelium, bundles of microfilaments course through the terminal web, terminating at specialized membrane regions called the zonula adherens (4). Microfilament bundles in smooth muscle cells appear to be much less regularly organized than in either striated muscle or epithelial brush border but are known to terminate at the cell membrane at specialized sites called dense plaques (5). In cultured fibroblasts, microfilament bundles do not exhibit ordered arrangements and usually terminate at those sites on the ventral surface of the cell membrane where the cell forms strong but transient attachments (focal adhesions) to the substratum (6).

It has recently been shown in this laboratory (7) that a 130,000 molecular weight intracellular protein isolated from chicken gizzard smooth muscle is present in cultured chicken embryo fibroblasts and, by immunofluorescence observations, seems to be concentrated on the cytoplasmic surface of the cell membrane coincident with the sites of focal adhesions to the substratum. This protein, which has been named "vinculin" (8), is probably the same protein that others have observed (9–11), but no specific location or proposed function had previously been ascribed to it. The observations with fibroblasts suggested, however, that vinculin might be more widely involved in the attachment of the termini of microfilament bundles to membranes, and this possibility was explored in the studies reported herein. These studies have been made with the intestinal brush border, gizzard smooth muscle, and cardiac striated muscle from chicken because these tissues contain the microfilament–membrane attachment sites mentioned above. Using immunoferritin electron microscopic labeling of vinculin on ultrathin frozen sections (12–14), we have found that vinculin is indeed sharply localized close to the membrane at all of these different microfilament–membrane attachment sites. These results suggest, therefore, that vinculin may play an important and widespread role in mediating such attachments. Some of these results have been briefly reported (8).

MATERIALS AND METHODS

Affinity-purified rabbit antibodies to chicken gizzard vinculin and affinity-purified goat antibodies to rabbit IgG were prepared as described (7). Ferritin conjugates of the goat antibodies were prepared by the method of Kishida *et al.* (15). Chicken small intestine, chicken gizzard, and chicken heart apex were dissected to blocks 1 mm square or smaller in a fixative solution containing 3% paraformaldehyde and 20 mM ethylacetimidate in phosphate-buffered saline at pH 7.4 (13). After incubation for 2–10 min, the blocks were transferred into 3% paraformaldehyde/0.1% glutaraldehyde. This two-stage fixation procedure was required because vinculin loses its capacity to bind antibody when treated directly with glutaraldehyde-containing solutions (unpublished results). After 1 hr in the second-stage formaldehyde-glutaraldehyde fixative, the specimens were rinsed, infused with 0.6 M sucrose, and rapidly frozen in liquid N_2. Ultrathin frozen sections were cut as described (12–14). The rabbit antibodies to vinculin were applied to the thawed sections at 50–100 μg/ml for 10 min at room temperature. After thorough rinsing, the sections were treated with the ferritin-conjugated goat antibodies to rabbit IgG at 50–200 μg/ml. Light positive staining of the immunolabeled sections was carried out

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as described (16). Control experiments were carried out in which normal rabbit IgG was substituted for the rabbit antibodies to vinculin. Sections were examined in a Philips model 300 transmission electron microscope operated at 60 kV.

RESULTS

In intestinal epithelial brush border, specific indirect immunoferritin labeling for vinculin was confined to the region close to the membrane of the zonula adherens (bracket 2 in Fig. 1 *A* and *B*) of the junctional complex formed between adjacent epithelial cells. No labeling of the tight junctions (brackets 1 in Fig. 1, *A* and *B*), the spot desmosomes (bracket 3 in Fig. 1*B*), the terminal web apart from the zonula adherens, or the microvilli (bracket V in Fig. 1*A*), including their tips (not shown), was observed. Control experiments using normal rabbit IgG in place of the rabbit antibodies to vinculin showed no significant ferritin labeling (Fig. 1*C*).

With gizzard smooth muscle, the immunoferritin labeling for vinculin was concentrated close to the cell membrane where the dense plaques were located (arrows in Fig. 2 *A* and *B*). Adjacent cell membrane regions that were not associated with dense plaques (region between two white arrowheads in Fig.

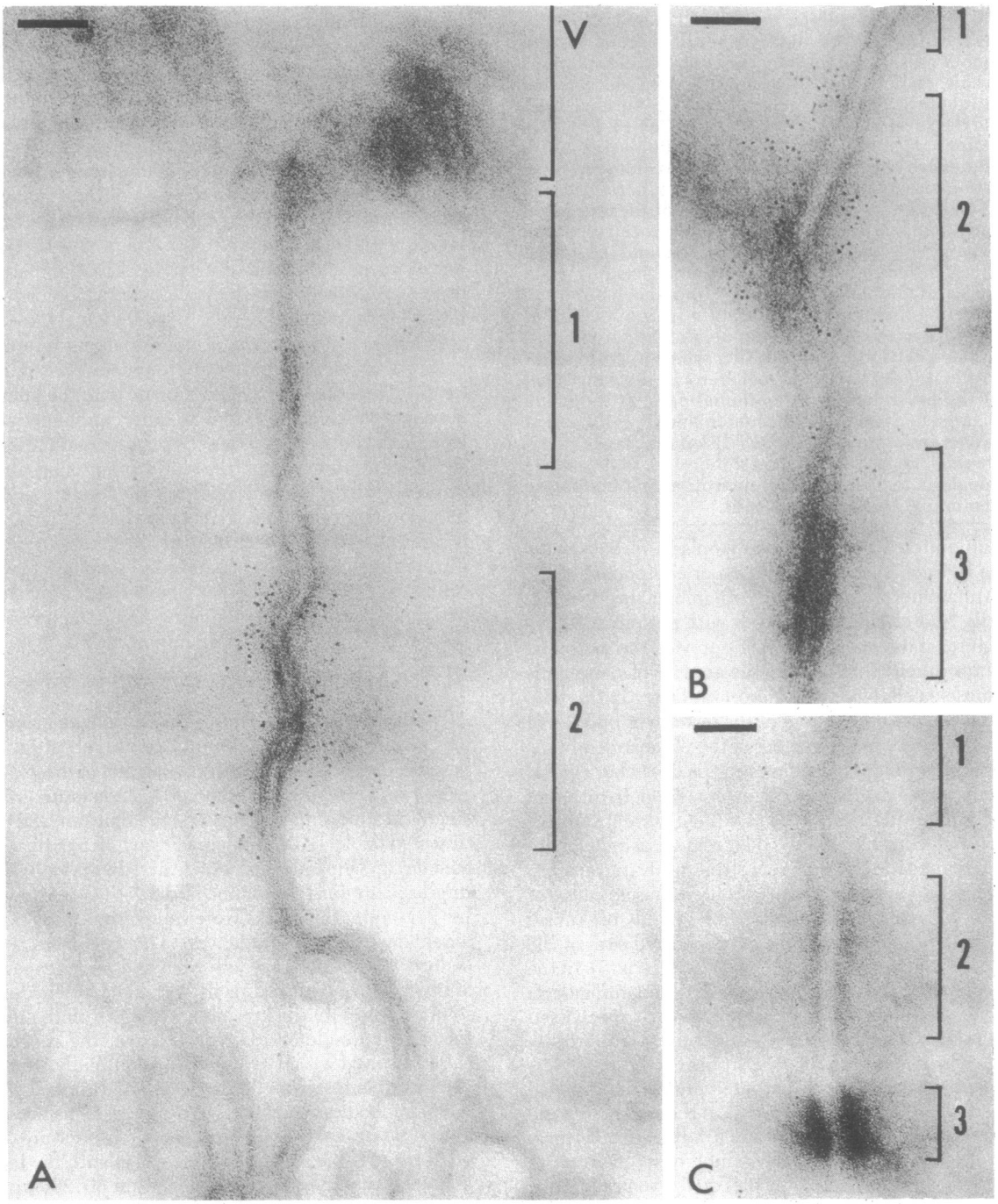

FIG. 1. Indirect immunoferritin labeling of chicken intestinal brush border for vinculin. In *A* and *B*, the ferritin label is localized at the zonula adherens (bracket 2) but not at the tight junction (bracket 1), the desmosome (bracket 3 in *B*), or microvilli (bracket V in *A*). In *C*, the control, the section was first treated with normal rabbit IgG and then with ferritin-labeled goat anti-rabbit IgG. No significant ferritin labeling is found at the zonula adherens (bracket 2) or other junctional structures (brackets 1 and 3). In these and subsequent micrographs, scales indicate 0.1 μm.

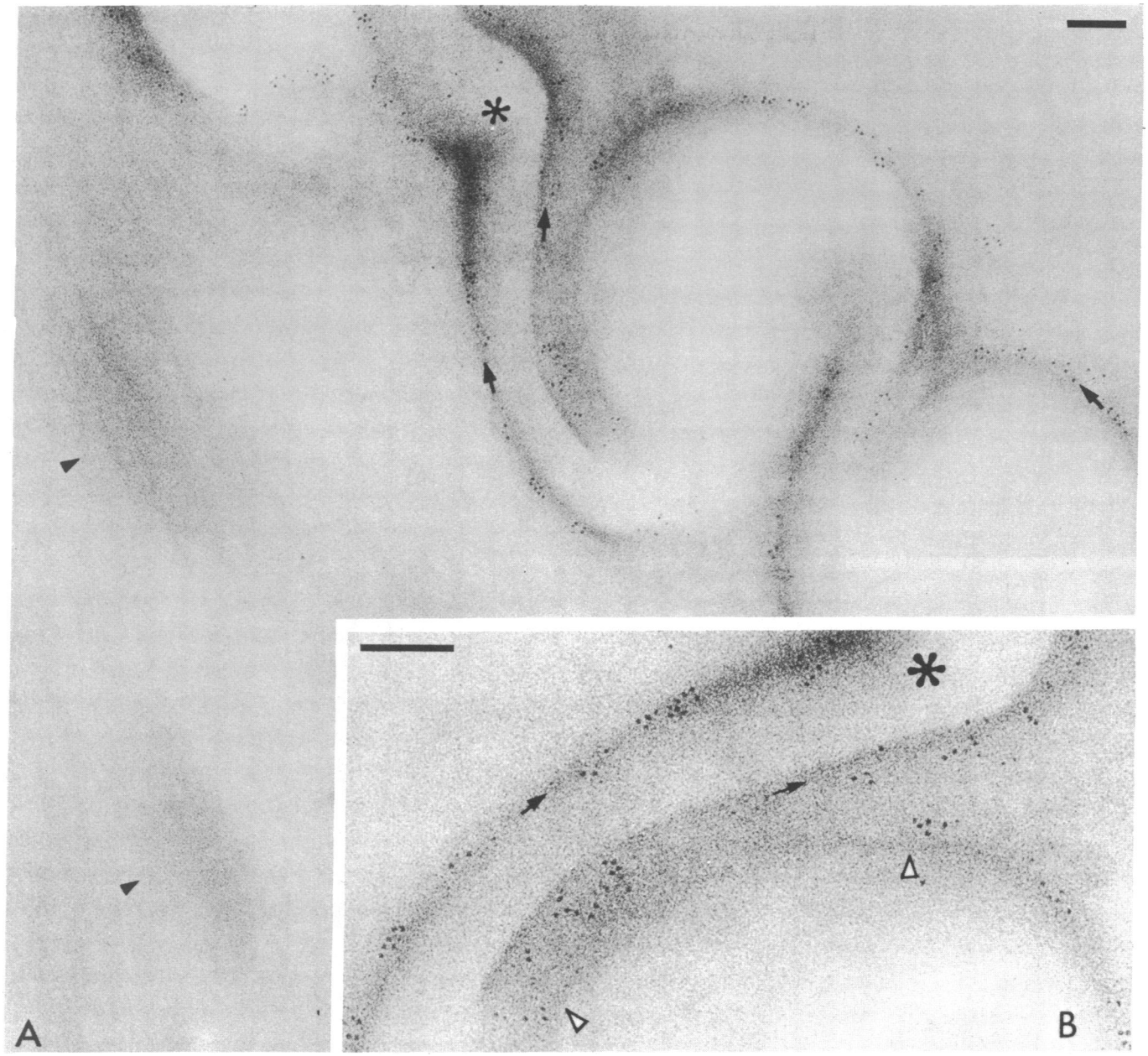

FIG. 2. Indirect immunoferritin labeling of chicken gizzard for vinculin. *B* is an enlarged portion of *A*. The convolutions of the cell membranes result from muscle contraction. Ferritin labeling is localized at the dense plaques associated with the cell membranes (arrows mark the plaques and asterisks mark the intercellular spaces). Labeling is not found in the cytoplasmic dense bodies (arrowheads in *A*).

2*B*) were not labeled. Significant labeling was absent from the cytoplasm and, in particular, was not found on the cytoplasmic dense bodies (black arrowheads, Fig. 2*A*).

In the myocardium, immunoferritin labeling for vinculin was localized close to the transverse portions of the intercalated disk membranes (Fig. 3*A*) at the fascia adherens where the actin microfilaments terminated. No significant labeling was observed near adjacent transverse portions of the intercalated disk membranes, the desmosomes, or macula adherens, which are known to be associated with intermediate filaments (D in Fig. 3*B*); or near contiguous longitudinal portions of the same membrane where the tight junctions (macula occludens) were located (O in Fig. 3*B*). No significant labeling for vinculin was found elsewhere within the sarcomere; in particular, the Z line showed no labeling (Fig. 3*C*).

DISCUSSION

Vinculin is an intracellular protein which was recently isolated from chicken gizzard smooth muscle and shown by immunofluorescence to be associated with the focal adhesions formed between cultured fibroblasts and their substrata (7). It is probably the same as the protein observed by others (9–10) with which, however, no function was associated previously. The present results show that vinculin, or a protein antigenically closely related to it, is present in a range of different cell types and, in each case, is sharply localized close to membrane sites where bundles or arrays of microfilaments terminate. Vinculin is absent from other regions of the same cell membranes, including other specialized junctional elements. These results lend support to the proposal (7) that the function of vinculin may be to participate in the linkage of the termini of microfilament bundles to membranes (*vinculum* ≡ link, Lat.).

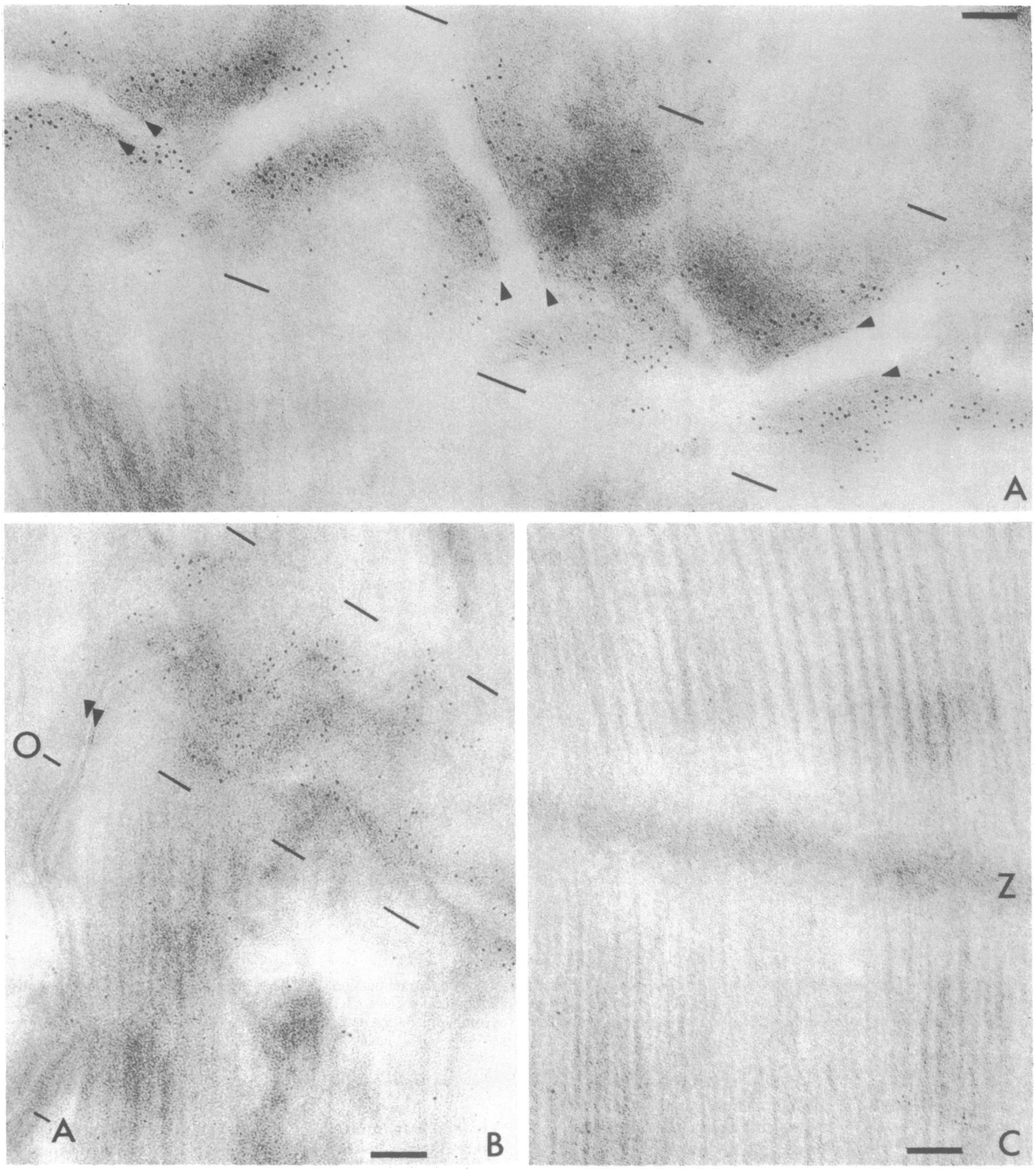

FIG. 3. Indirect immunoferritin labeling of chicken cardiac muscle for vinculin. The ferritin label is localized in the fascia adherens of the intercalated disks (the areas enclosed by a pair of broken lines in *A* and *B*). The sectional plane was nearly perpendicular to the disk membranes in *A*, thus revealing profiles of the membranes at some sites (arrowheads in *A*); but sectioning was oblique in *B*. Labeling is not found at the macula occludens (O in *B*: arrowheads indicate closely apposed adjacent cell membranes) or at the desmosome (D in *B*). No significant labeling is seen at the Z line (Z in *C*) in an adjacent portion of the same specimen.

The protein α-actinin has previously been implicated in the linkages of microfilament bundles to membranes (17). Immunoperoxidase experiments have indicated that α-actinin is associated with both cytoplasmic dense bodies and membrane-localized dense plaques in smooth muscle (18), and a concentration of immunoferritin labeling for α-actinin near the zonula adherens in intestinal epithelial brush border (16) has been observed. Furthermore, both α-actinin (7, 19) and vinculin (7) were found by immunofluorescence to be concentrated at the focal adhesion plaques formed by cultured fibroblasts in contact with substrata. However, in immunoelectron microscopic labeling experiments for both vinculin and α-actinin and, in

particular, in double immunolabeling experiments using ferritin–antibody and Imposil–antibody conjugates simultaneously (20), we have found that the labeling for vinculin is in closer proximity to the membranes of both the zonula adherens of intestinal epithelial brush border and the membrane-associated dense plaques of smooth muscle than is the labeling for α-actinin (unpublished results). This closer proximity to the membrane at these sites suggests that vinculin may play a more direct role than α-actinin in the linkage of the termini of microfilament bundles to membranes.

If indeed a common type of linkage is involved in the cases we have studied, the molecular details of such a linkage are unknown. Vinculin is a soluble protein, and there is no evidence that it is itself an integral protein of the cell membrane. It might therefore attach to the termini of individual microfilaments, collect them into bundles, and thus indirectly promote the binding of the termini to some integral protein(s) of the membrane; or it might itself serve to link the microfilament termini directly or indirectly to such integral protein(s).

The frequent finding that vinculin is localized close to sites of termination of microfilament bundles at membranes does not mean that vinculin is involved in *all* such linkages. For example, bundles of microfilaments terminate at membrane sites at the tips of the microvilli of intestinal brush border (17), but no vinculin labeling was found close to those sites on the same specimens that showed vinculin labeling at the zonula adherens. Recent evidence indicates that α-actinin is also absent from those sites (16, 21, 22). These results suggest therefore that there exists within microvilli a different type of attachment of microfilament termini to membranes than the type involving vinculin.

Microfilaments or thin filaments are also known to be associated with specialized nonmembranous sites inside cells, such as the cytoplasmic dense bodies of smooth muscle cells (23) and the Z line in striated muscle (cf. ref. 3). Our immunolabeling results show, however, that vinculin is not associated with these intracellular sites, which are thereby distinguished from the vinculin-containing sites where microfilament termini are attached to membranes.

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