Functional Studies of the Domains of Talin

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Abstract. The protein talin has two domains of ~200 and 47 kD, which can be cleaved apart by a variety of proteases. To examine the function of these two structural domains of talin, we have digested purified talin with a calcium-dependent protease and separated the resulting fragments chromatographically. Both fragments were radioiodinated and used to probe Western blots of whole fibroblasts and chicken gizzard extracts. The large talin fragment bound to vinculin and metavinculin. The small fragment did not demonstrate any binding in this assay. The fragments were labeled fluorescently and microinjected into fibroblasts in tissue culture. The large talin fragment incorporated quickly into focal adhesions where it remained stable for at least 14 h. The small fragment associated with focal adhesions of fibroblasts but was also distributed diffusely in the cytoplasm and the nucleus. These experiments suggest that talin has at least two sites that contribute to its localization in focal adhesions. Intact talin microinjected into Madin-Darby bovine kidney epithelial cells localized to the focal adhesions but was excluded from the zonulae adherentes, despite the localization of vinculin to both of these sites. In contrast, the large talin fragment, when microinjected into these epithelial cells, incorporated into both focal adhesions and zonulae adherentes. The difference in localization between the large talin fragment and intact talin seems to be due to the removal of the small domain. This difference in localization suggests that talin binding sites in zonulae adherentes have limited accessibility.

FIBROBLASTS in tissue culture adhere to the surface on which they are grown via discrete regions where their ventral plasma membranes come in close proximity to the substrate. The membrane at these sites contains a concentration of extracellular matrix (ECM)[1] receptors, and there is a dense protein plaque that is associated with the cytoplasmic face of this membrane region. ECM proteins associate with the external face of the membrane at these sites, and the whole structure including cytoplasmic, membrane, and external domains is termed a focal contact or focal adhesion (for review see Burridge et al., 1988). The cytoskeletal protein talin localizes to these sites (Burridge and Connell, 1983) where actin filaments, bundled into stress fibers, terminate and anchor to the plasma membrane. Besides talin and actin, there are a number of other proteins that are enriched in focal adhesions such as vinculin (Geiger, 1979; Burridge and Feramisco, 1980), α-actinin (Lazarides and Burridge, 1975), and members of the integrin family of transmembrane, ECM receptors (Damsky et al., 1985; Chen et al., 1985; Kelly et al., 1987; Marcantonio and Hynes, 1988). In view of their colocalization, these proteins are suspected participants in linking actin filaments through the membrane to the ECM or substratum.

Epithelial cells in tissue culture exhibit two types of specialized membrane regions where actin filaments associate with and terminate at the plasma membrane. Many epithelial cells in tissue culture have focal adhesions which appear identical in composition to those seen in fibroblasts. Epithelial cells both in vivo and in tissue culture interact with neighboring cells in specialized regions of the plasma membrane (Farquhar and Palade, 1963), which we will refer to as zonulae adherentes (ZA). These structures have also been called intermediate junctions or belt desmosomes and are characterized by a contractile bundle of actin filaments that is associated with the plasma membrane at the cell's perimeter near the apical surface. ZA, being sites of concentration of actin filaments, α-actinin, and vinculin (Geiger et al., 1981), appear similar in composition to focal adhesions, although there are some striking differences. Talin, for example, while present in the focal adhesions of epithelial cells, is absent from ZA in the same cells (Geiger et al., 1985). The structures also differ in integral membrane protein composition. Focal adhesions are sites where integrins are concentrated, but ZA are enriched in members of the family of cell adhesion molecules (CAMs) (Damsky et al., 1983; Volk and Geiger, 1984, 1986; Behrens et al., 1985; Boller et al., 1985; Gumbiner and Simons, 1986; Hatta and Takeichi, 1986). Unlike focal adhesions which are cell-to-substratum associations, ZA are cell-to-cell associations and the pre-

1. *Abbreviations used in this paper*: CDPII, calcium-dependent protease; CEF, chicken embryo dermal fibroblast; ECM, extracellular matrix; IATR, iodoacetamidotetramethyl rhodamine; IRM, interference reflection microscopy; MDBK, Madin-Darby bovine kidney; PBS+, PBS with calcium and magnesium; REF52, a rat embryo cell line; ZA, zonulae adherentes.

dominant form of membrane glycoprotein at each of these sites reflects this difference. The coexistence of talin and integrin in focal adhesions and their concomitant absence from ZA is consistent with an interaction between these two proteins.

Biochemical assays have demonstrated that talin can interact with both vinculin (Burridge and Mangeat, 1984) and integrin (Horwitz et al., 1986) in vitro. Vinculin binds α-actinin in Western blot overlays (Otto, 1983; Wilkins et al., 1983; Burridge and Mangeat, 1984; Belkin and Koteliansky, 1987) and in binding assays in solution (Wachsstock et al., 1987). α-Actinin cross-links actin filaments in vitro (Maruyama and Ebashi, 1965; Podlubnaya et al., 1975). This cross-linking is thought to contribute to the formation of stress fibers in living cells (Lazarides and Burridge, 1975; Bennett et al., 1984). The data from these biochemical assays provide a possible set of links between actin and the ECM, although it is likely that other proteins aid in this or parallel sets of links between stress fibers and the ECM. It is probable that the function of vinculin and α-actinin in focal adhesions is similar to their function in ZA. The difference in membrane glycoproteins found at these two sites along with the absence of talin from ZA may suggest the existence of some other protein serving as a link between CAMs and vinculin.

The initial goal of this study was to characterize the function of talin's structural domains. Talin, purified from chicken gizzard smooth muscle (Molony et al., 1987) or from human platelets (Collier and Wang, 1982; O'Halloran et al., 1985; Turner and Burridge, 1989), is a high molecular weight protein with an elongated morphology at physiological ionic strength (Molony et al., 1987). Both forms of talin can be cleaved by a calcium-dependent protease (CDPII) into two stable fragments (Fox et al., 1985; O'Halloran et al., 1985; Beckerle et al., 1987), which we will refer to as the large fragment and the small fragment. These two stable fragments represent distinct domains of the molecule, and a number of other proteases cleave talin to produce similar sized fragments (O'Halloran et al., 1985; Beckerle et al., 1986). A significant portion of the talin identified in aggregated platelets is in the form of these two fragments, although the physiological significance of this cleavage is not clear (Fox et al., 1985). The presence of CDPII in the focal adhesions of fibroblasts and Madin-Darby bovine kidney (MDBK) cells suggests that focal adhesion stability may be regulated by increased levels of calcium resulting in proteolytic cleavage of talin (Beckerle et al., 1987). However, disruption of focal adhesions by a variety of chemical agents has been shown to occur without detectable proteolysis of talin (Turner et al., 1989).

In this study, we describe the digestion of purified talin in vitro with CDPII, the separation of the two domains, and experiments with each of these protein fragments aimed at determining their properties. We have radioiodinated each of the purified fragments of talin and used Western blot overlays as an assay for in vitro interaction of the two talin domains with polypeptides from whole cells. We have labeled fluorescently the purified fragments and have microinjected these into fibroblasts and MDBK cells, an epithelial cell line known to form ZA. These microinjection experiments allowed us to assay each domain for localization at sites of actin filament interaction with the plasma membrane.

Materials and Methods

Cell Culture and Microinjection

Cells of a rat embryo cell line (REF52) were maintained in DME containing 15 mM Hepes, pH 7.3, 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (pen/strep). Chicken embryo dermal fibroblasts (CEFs) were obtained from 8–12-d-old embryos and were grown in the same medium as REF52 cells. Cells were passaged onto 22-mm round glass coverslips 24 h before microinjection. Cells were at subconfluent densities at the time of injection. MDBK cells were maintained in Eagle's Minimum Essential Medium with Earle's salts (EMEM), 15 mM Hepes, pH 7.3, 10% FBS, and pen/strep. These cells were passaged onto the glass coverslips 24–48 h before microinjection at a density that provided small groups of cells (<50) with lateral junctions at the time of injection. Large islands of confluent cells did not stain well and were too thick for optimal immunofluorescence.

Coverslips were removed from their normal growth medium and a small circle was scored in the center of the coverslip to facilitate subsequent location of the injected cells. For control injections with fluorescently labeled BSA or ovalbumin, a square was scored into the coverslip in addition to the circle so that experimental and control cells were on the same coverslip and received the same postinjection treatment. The coverslips were transferred to freshly gassed medium for microinjection which contained 15 mM Hepes and pen/strep in DME or EMEM depending on the cell type. Injections were performed at room temperature. Cells on coverslips were microinjected with unlabeled whole chicken gizzard talin, chicken gizzard vinculin, or fluorescently labeled fragments of chicken or human talin using a glass capillary (Kwik-fil; World Precision Instruments, Inc., New Haven, CT) pulled on a micropipette puller (Brown-Flaming; Sutter Instrument Co., San Francisco, CA). Microinjection was performed on a Diavert microscope using a 32× phase objective and a micromanipulator (E. Leitz, Inc., Rockleigh, NJ). After most of the cells in the scored circle had been injected (~30 min), each coverslip was returned to the incubator in its normal FBS-containing medium. Postinjection incubations were from 0 to 14 h.

For one of the microinjection experiments with the small talin fragment (described below), coverslips were first incubated with 50 μg/ml human plasma fibronectin (New York Blood Center, NY) in PBS with calcium and magnesium (PBS+) for 30 min at 37°C followed by a brief wash in PBS+. REF52 cells were trypsinized off the culture dish and plated onto these coated coverslips in complete medium. After 30 min of incubation, these cells, which had just begun to spread, were microinjected. The cells were then returned to the incubator to complete spreading over the course of 6 h.

Talin and Vinculin Purification

Talin was purified from outdated human platelets by the method of Turner and Burridge (1989). Talin and vinculin were purified from chicken gizzard as described by Molony et al. (1987) or Feramisco and Burridge (1980), respectively. For experiments in which cells were microinjected with intact, unlabeled talin or vinculin, these proteins were taken from the peak fractions eluted from the final chromatography columns used for their respective purifications and dialyzed into microinjection buffer (75 mM KCl, 0.1% β-mercaptoethanol, 10 mM potassium phosphate, pH 7.5). Intact talin was used for microinjection at a concentration comparable to that of fluorescently labeled large talin fragment, as judged by Coomassie blue staining of electrophoresed polypeptides. Vinculin was used for microinjection at ~1 mg/ml.

Proteolytic Cleavage

Purified talin either from chicken gizzard or human platelets was cleaved by CDPII (a gift of Dr. D. Croall, University of Texas Southwestern Medical Center, Dallas, TX) at room temperature. Talin in buffer B (20 mM NaCl, 0.1 mM EDTA, 0.1% β-mercaptoethanol, 20 mM Tris-acetate pH 7.6) plus 1 mM EGTA was digested with CDPII at a final talin to enzyme molar ratio of 500:1. To start the reaction, 1 M $CaCl_2$ was added to the reaction mixture to a final concentration of 8 mM. After 30 min of digestion, 250 μg of leupeptin (Sigma Chemical Co., St. Louis, MO) was added to the digestion mixture and the products were separated by chromatography on an FPLC anion exchange column (mono Q, Pharmacia Fine Chemicals, Piscataway, NJ) eluted with a gradient of buffer B to buffer B plus 400 mM NaCl.

Fluorescent Labeling of Talin Fragments

Purified large talin fragment was conjugated with either FITC (Organics

Research Inc., Cleveland, OH) or iodoacetamidotetramethyl rhodamine (IATR; Molecular Probes Inc., Junction City, OR) and the small fragment was labeled with FITC. Labeling of the small talin fragment with IATR caused this polypeptide to aggregate and clog microinjection needles. For the FITC labeling, ~3 ml of purified talin fragment in buffer B at ~0.8 mg/ml was dialyzed overnight against 200 mM sodium carbonate/bicarbonate (C/B) buffer, pH 9.0. The dialysis bag was then transferred to 200 ml of the C/B buffer to which 6 mg FITC was added with stirring. For the IATR labeling, a similar amount of protein off the mono Q column was dialyzed overnight against 200 mM sodium borate buffer, pH 7.5. 1 mg of IATR was dissolved in 10 μl of DMSO, and this solution was diluted 1:10 in the borate buffer before adding a total of 22 μg of dissolved IATR to the protein solution. In both conjugations, the protein fragment was incubated with the dye for 6–8 h. The IATR conjugation was stopped by bringing the reaction mixture to 0.1% DTT. Free dye was removed by dialysis for several days against microinjection buffer with changes every 12 h. The FITC- or IATR-talin fragments were concentrated by dialysis against 75% wt/vol sucrose in microinjection buffer plus 0.1% NaN_3 for ~3 h. The bag was retied at the smaller volume and dialyzed overnight against microinjection buffer. In some cases, the dialysis tube containing the protein solution was incubated with dry Sephadex G-50 until the volume of the solution reached 50 μl or less (~3 h). The bag was retied and dialyzed overnight against 2 liters of microinjection buffer. Aliquots of the labeled large talin fragment could be frozen in liquid N_2 and stored at −70°C for several months with no noticeable change in behavior after injection. The small talin fragment was stored at 0–4°C until use, and used within 1 mo of preparation.

To determine the concentration of talin fragments in solution and so to determine the dye to protein ratio, a molar extinction coefficient was calculated using the amino acid composition for each fragment as determined by Dr. D. J. G. Rees (personal communication) and a formula described by Edelhoch (1967).

Immunostaining and Microscopy

After the postinjection incubation, the coverslips were fixed in 3.7% formaldehyde in PBS+ for 5–10 min, and then permeabilized with 0.2% Triton X-100 in PBS+ for 5 min, followed by several washes in PBS+. MDBK cells were fixed and permeabilized simultaneously in 3.7% formaldehyde, 0.2% Triton X-100 in PBS+ for 5 min. These cells were then washed briefly in PBS+ followed by a 15-min incubation in normal goat serum diluted 1:20 in PBS+ containing 5 mg/ml BSA and 0.02% NaN_3. For some experiments, fixed, permeabilized fibroblasts were incubated in rhodamine phalloidin (Molecular Probes Inc.) at 7 nM in PBS+ for 10 min followed by several washes with PBS+.

Immunostaining for talin was performed using two different mouse anti-talin mAbs characterized previously (Otey et al., 1990). One of them, 8d4, recognizes talin from a wide variety of species including murine, bovine, and avian, whereas the other, 3f5, only recognizes avian talin. All monoclonals were used as hybridoma cell supernatants in DME with 15% FBS, pen/strep, and 0.1% NaN_3. Either FITC- or rhodamine isothiocyanate (RITC)-goat anti-mouse IgG (both from Cappel Laboratories, Malvern, PA) was used as a secondary antibody diluted 1:50 in PBS+.

Polyclonal guinea pig antivinculin has been characterized previously (Turner et al., 1989) and was used at 1:100 dilution. FITC goat anti-guinea pig (Cappel Laboratories) was diluted 1:100 in PBS+. A mouse monoclonal that binds to chicken vinculin, but not to vinculin in MDBK cells, was used as hybridoma cell supernatant. Polyclonal rabbit anticanine uvomorulin was kindly provided by Dr. Barry Gumbiner (University of California at San Francisco) and diluted 1:200. FITC-goat anti-rabbit (Cappel Laboratories) was used at 1:50 or RITC-donkey anti-rabbit, species cross-adsorbed (Cappel Laboratories) was used at 1:100.

After fixation and permeabilization, coverslips were inverted onto a 50-μl drop of antibody and incubated in a humid chamber for 30 min at 37°C. For double labeling, secondary antibodies were combined into one solution with each antibody at the dilution it would have been used at individually. After a final wash, the coverslips were rinsed in deionized water to remove salts and mounted in 10% Mowiol (Calbiochem-Behring Corp., San Diego, CA) in a Tris buffer, pH 8.5, and air dried. For interference reflection microscopy (IRM), a chamber was constructed consisting of a glass slide with a 16-mm hole in it with a coverslip mounted on one side with wax. The chamber was filled with a solution of 50% glycerol, 0.1% NaN_3 in PBS+ with the pH adjusted to 8.5 using NaOH. The coverslip with fixed cells on it was inverted and sealed onto the slide chamber with silicon grease. Microscopy was performed on an IM35 (Carl Zeiss, Inc., Thornwood, NY). Fluorescence was photographed through a Zeiss Planapo 63× objective on TMAX 400 or P3200 film processed to 800–1600 ASA with TMAX developer (Eastman Kodak Co., Rochester, NY). IRM images were photographed through a Zeiss Antiflex 63× objective on Kodak Tech Pan 2415 film developed with DI9 to 125 ASA.

Some of the MDBK cells were flat enough for both the focal adhesions of the ventral plasma membrane and the ZA near the apical surface of the cell to be in focus at the same time. Other cells were too tall for both of these structures to be in focus in the same photograph and so in these cases both focal planes of interest were photographed.

Iodination and Western Blot Overlays

All procedures for iodination of talin fragments, gel electrophoresis, Western blotting, and overlays were performed as described elsewhere (Turner and Burridge, 1989).

Results

Chicken gizzard talin and human platelet talin were purified and cleaved with CDPII. The products of these digestions were subjected to FPLC anion exchange chromatography to separate the different polypeptides in the cleavage mixture (Turner and Burridge, 1989). In most digestions, no talin was left undigested. The small fragment (Fig. 1, lane *2*) eluted from the mono Q column as the elution buffer reached ~140 mM NaCl. The large fragment (Fig. 1, lane *3*; the chicken form is shown) eluted from the column at ~320 mM NaCl. The fragments were labeled either with ^{125}I or fluorescent dyes as described in Materials and Methods. The fluorescent form of each of these fragments was analyzed by SDS-PAGE (Fig. 1, lanes *2′* and *3′*). The FITC-labeled small talin fragment had a dye to protein molar ratio of 1.3 and was used for microinjections at ~3 mg/ml. The IATR large fragment had a dye to protein molar ratio of 0.9 and was microinjected at a concentration of ~4.5 mg/ml.

Talin Fragments in Western Blot Overlays

The iodinated talin fragments were used to overlay Western blots of whole rat fibroblasts and fractionated chicken gizzard to identify polypeptides with which these fragments in-

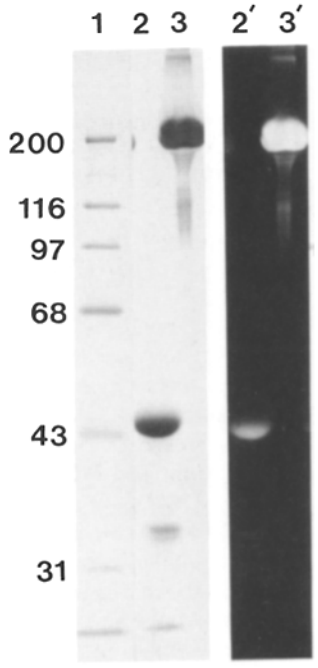

Figure 1. Fluorescent labeling of talin fragments. Purified chicken gizzard talin was cleaved by CDPII. The two resulting fragments were separated from one another and from any remaining intact talin by anion exchange chromatography. The small talin fragment (lanes *2* and *2′*) was labeled with FITC. The large fragment (lanes *3* and *3′*) was labeled with FITC or IATR (IATR form shown). Lanes *2* and *3* show the Coomassie blue-stained gel of the fluorescently labeled fragments, and lanes *2′* and *3′* show the fluorescence emitted from lanes *2* and *3*. Each of the fragments have similar dye to protein ratios, but the apparent intensity difference in this figure is due to the photographic filter used. Lane *1*, molecular mass standards (kilodaltons).

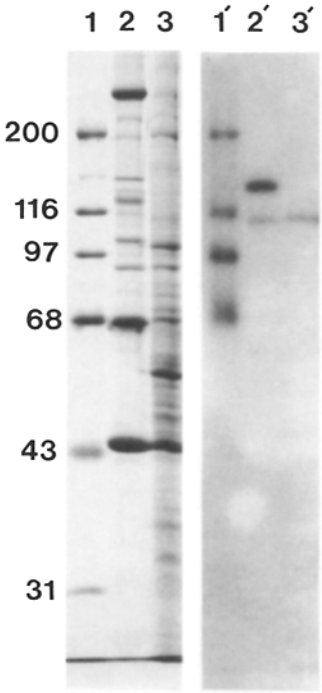

Figure 2. ^{125}I-large talin fragment in Western blot overlays. A fraction from early in the purification of chicken gizzard talin (lane *2*) and whole rat embryo fibroblasts (REF52) (lane *3*) were electrophoresed on a 10% polyacrylamide SDS gel and stained with Coomassie blue. A Western blot of an identical gel was incubated with radioiodinated large talin fragment. The autoradiograph shows that this fragment binds to vinculin (116 kD) and metavinculin (150 kD) in the gizzard extract (lane *2′*) and to vinculin in fibroblasts (lane *3′*). Lane *1* molecular mass standards and lane *1′* ^{125}I-labeled standards (kilodaltons).

teract. The autoradiographs demonstrated that the large talin fragment interacts strongly and consistently with a 116-kD band in both samples (Fig. 2, lanes *2′* and *3′*) and with an additional 150-kD band found in the chicken gizzard extract (Fig. 2, lane *2′*). These observations are consistent with data presented for the whole talin molecule on blots of CEF and gizzard extract (Burridge and Mangeat, 1984). The 116-kD band is vinculin and the larger band is an isoform of vinculin known as metavinculin (Feramisco et al., 1982; Siliciano and Craig, 1982). The small fragment of the human or the chicken talin exhibited no consistent interaction in blots of gizzard preparations, or chicken or rat fibroblasts (data not shown).

Microinjection of Intact Talin into Fibroblasts

Purified, intact, unlabeled chicken gizzard talin was microinjected into REF52 cells followed by a 2-h postinjection incubation. The injected talin was selectively localized in these cells using an mAb specific for avian talin. Fig. 3 *a* shows the immunolocalization of the chicken talin in such an experiment. The exogenous talin was concentrated into focal adhesions (*arrowheads*) the positions of which were confirmed by IRM (Fig. 3 *b*). Another cell in the field that had not been microinjected was visible in the IRM image but not in the fluorescence image, confirming that the monoclonal antibody used to detect the injected chicken talin does not recognize the talin endogenous to these cells.

Microinjection of the Large Talin Fragment into Fibroblasts

The large fragment of human talin labeled with FITC, or the large fragment of chicken talin labeled with IATR was injected into CEF or REF52 cells. Similar results were obtained with each of these reagents, indicating that the localization of the large fragment was independent of the species of talin used or the type of fluorophore. Fig. 4 shows the localization of fluorescently labeled large fragment (chicken) 3 h after microinjection into REF52. As with the localization of whole talin injected into these cells, there was a strong accumulation of the large talin fragment into focal adhesions (Fig. 4, *a* and *c*) the positions of which were confirmed by IRM (Fig. 4, *b* and *d*). The adhesions appeared normal in terms of number, closeness of the membrane to the substratum, and location within the cell. All contacts appeared equally labeled with the probe, and the probe was evenly distributed within each contact. Fig. 4, *c* and *d* show two REF52 cells, one of which had been injected with the fluorescent large talin fragment 2 h before fixation. The IRM image (Fig. 4 *d*) showed several small focal contacts (*arrowhead*) in what appears to be a newly spread lamella. The fluorescence image (Fig. 4 *c*) confirmed that these presumptive immature adhesions (*arrowhead*) incorporated the exogenous large talin fragment like all other adhesions in the injected cell. The large talin fragment could be detected in

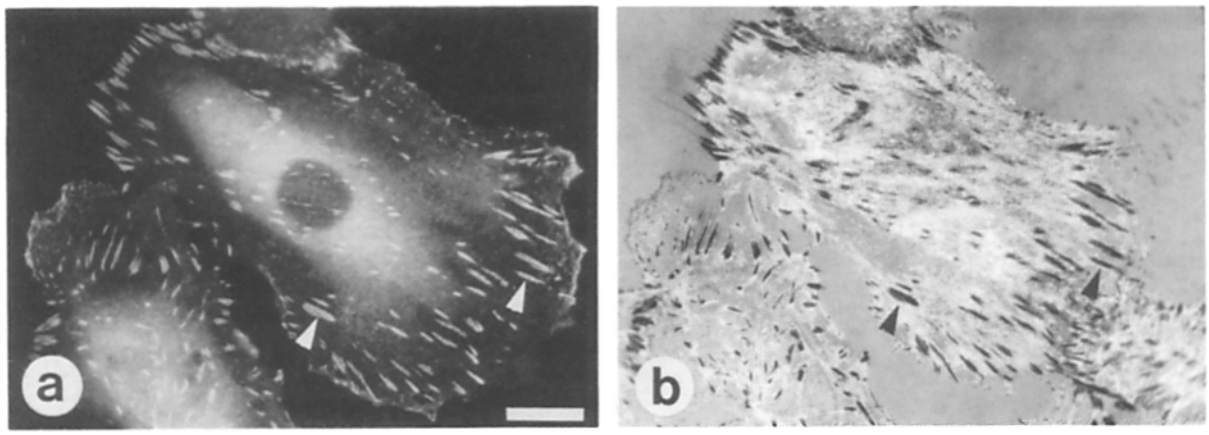

Figure 3. Intact chicken gizzard talin microinjected into fibroblasts. Purified, unlabeled talin was microinjected into REF52 cells followed by a 2-h incubation. The cells were immunostained using an mAb that is specific for avian talin (*a*). The microinjected talin incorporated into focal adhesions (*arrowheads*), the positions of which are confirmed by IRM (*b*). The antitalin mAb did not stain cells that had not been microinjected. Bar, 20 μm.

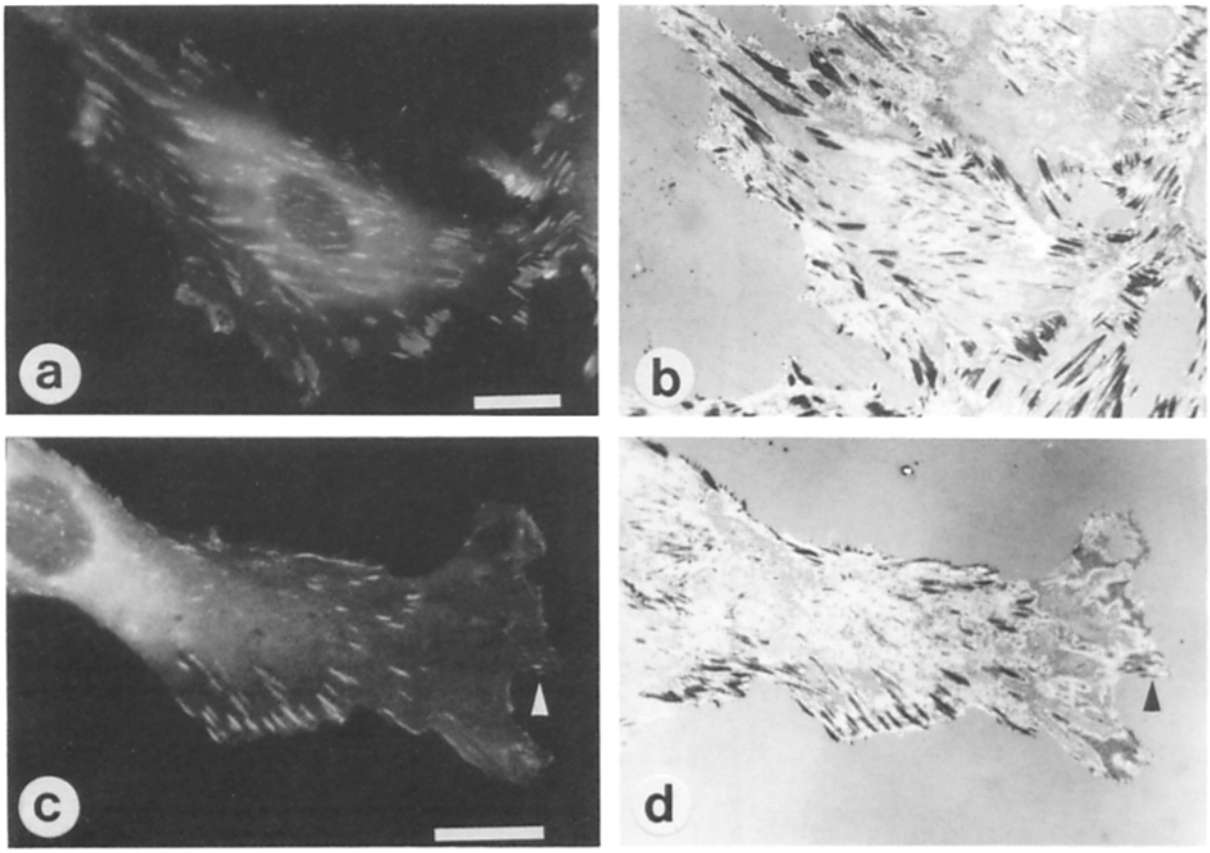

Figure 4. The large talin fragment microinjected into fibroblasts. The large talin fragment, labeled with FITC, was microinjected into REF52 cells followed by a 2-h incubation. The fragment accumulated into focal adhesions (*a* and *c*). The IRM images (*b* and *d*) showed that the focal adhesions of injected cells appeared identical to those of adjacent noninjected cells. The fluorescent large talin fragment even incorporated into small, presumptive immature focal adhesions (*arrowhead* in *c* and *d*). Bar, 20 μm.

focal adhesions in as little as 5 min after injection. Incorporation increased progressively up to ~2 h after injection (data not shown). The large talin fragment appeared to be stably incorporated into focal adhesions because it could be detected in these structures for at least 14 h after microinjection (data not shown). No incorporation into lysosomes was detected.

Microinjection of the Small Talin Fragment

The FITC-labeled small fragment of human or chicken talin also concentrated in focal adhesions several hours after microinjection into rat or chicken fibroblasts. Fig. 5, *a* and *c* show several focal adhesions (*arrowheads*) that have incorporated the human talin small fragment. These focal adhesions were confirmed by immunostaining for vinculin (Fig. 5 *b*) and IRM (Fig. 5 *d*). The majority of the probe, however, was distributed diffusely in the cytoplasm or localized to the nucleus. It should be noted that after microinjection under the same conditions, proteins such as rabbit immunoglobulins, fluorescently labeled BSA, or fluorescently labeled ovalbumin gave diffuse cytoplasmic localization in these cells with no labeling of focal adhesions (data not shown). In an attempt to enhance the incorporation of the small fragment into focal adhesions, the probe was microinjected into REF52 which had just begun to spread out on fibronectin-coated glass coverslips. The cells containing the fluorescent probe were allowed to spread out for over 6 h, during which time many new focal adhesions were formed. Despite these modifications to the protocol, no increase in focal adhesion localization resulted (data not shown). As with the large fragment, focal adhesions and stress fibers were not disrupted by the microinjection of the small talin fragment as shown by vinculin staining (Fig. 5 *b*), the corresponding IRM images (Fig. 5 *d*), or F-actin distribution (data not shown).

Immunofluorescent Localization of Talin, Vinculin, and Uvomorulin in MDBK Cells

MDBK cells were immunostained for vinculin, talin, and uvomorulin (L-CAM). Fig. 6 shows MDBK cells double labeled for vinculin (Fig. 6 *a*) and uvomorulin (Fig. 6 *b*). Consistent with previously published accounts of the localization of these proteins in epithelial cells (Geiger et al., 1985), vinculin localized to focal adhesions (*double arrowheads*) and to ZA (between *single arrowheads*), while uvomorulin localized only to ZA (between *single arrowheads*). Fig. 6 also shows MDBK cells double labeled for talin (Fig. 6 *c*) and uvomorulin (Fig. 6 *d*). Unlike vinculin, talin was found only in focal adhesions (*double arrowheads*) and there was no detectable talin staining in ZA as identified by uvomorulin staining (between *single arrowheads*).

Immunostaining of Western blots demonstrated that talin from these bovine epithelial cells comigrates on SDS polyacrylamide gels with human platelet talin but chicken giz-

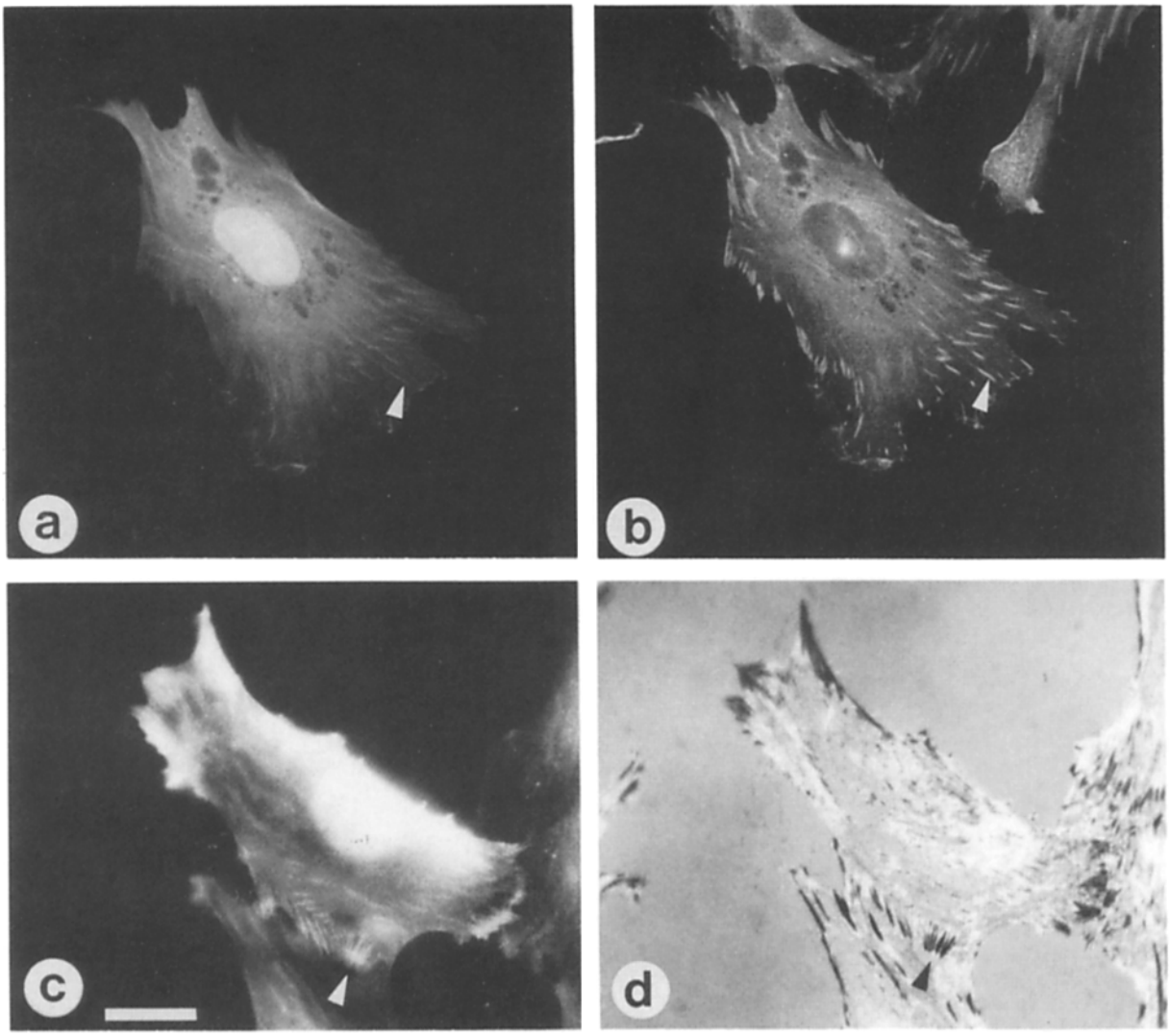

Figure 5. The small talin fragment microinjected into fibroblasts. The small talin fragment (FITC-labeled) was microinjected into CEF followed by a 2-h incubation. The probe labeled focal adhesions (*arrowheads* in *a* and *c*), the positions of which were confirmed by immunostaining for vinculin (*arrowhead* in *b*) and IRM (*arrowhead* in *d*). Much of the probe remained diffuse in the cytoplasm. Bar, 20 μm.

zard talin exhibits slightly faster electrophoretic mobility (data not shown). This suggests that the difference in molecular weight is due to species difference and not tissue type. ^{125}I-vinculin from chicken gizzard was used to overlay Western blots of whole MDBK cells where the probe bound to talin and the large talin fragment (data not shown).

Microinjection of Chicken Vinculin into MDBK Cells

Purified chicken gizzard vinculin was microinjected into MDBK cells followed by a 3-h postinjection incubation. The injected vinculin was localized using an mAb that binds to chicken vinculin but not to vinculin in mammalian cells. The injected vinculin localized to focal adhesions (Fig. 7 *a*, *double arrowheads*) and to ZA (Fig. 7 *a*, between *arrowheads*). Immunostaining demonstrated that the distribution of endogenous talin was not affected by vinculin microinjection, but continued to be localized to focal adhesions (Fig. 7 *b*, *double arrowheads*) and excluded from ZA.

Microinjection of Intact Talin into MDBK Cells

Purified, intact, unlabeled chicken gizzard talin was microinjected into MDBK cells followed by a 2-h postinjection incubation. The injected talin was selectively localized using an mAb specific for the chicken talin. Fig. 8 *a* shows the localization of microinjected intact talin to focal adhesions (*double arrowheads*) and its absence from ZA, which were identified by uvomorulin immunolocalization in the same cells (between *single arrowheads* in Fig. 8 *b*).

Microinjection of the Large Talin Fragment into MDBK Cells

The fluorescently labeled large fragment of talin from chicken or human was microinjected into MDBK cells followed by a 2-h postinjection incubation. Fig. 8 *c* shows that unlike intact talin, the large talin fragment localized to both focal adhesions (*double arrowheads*) and ZA (between *sin-*

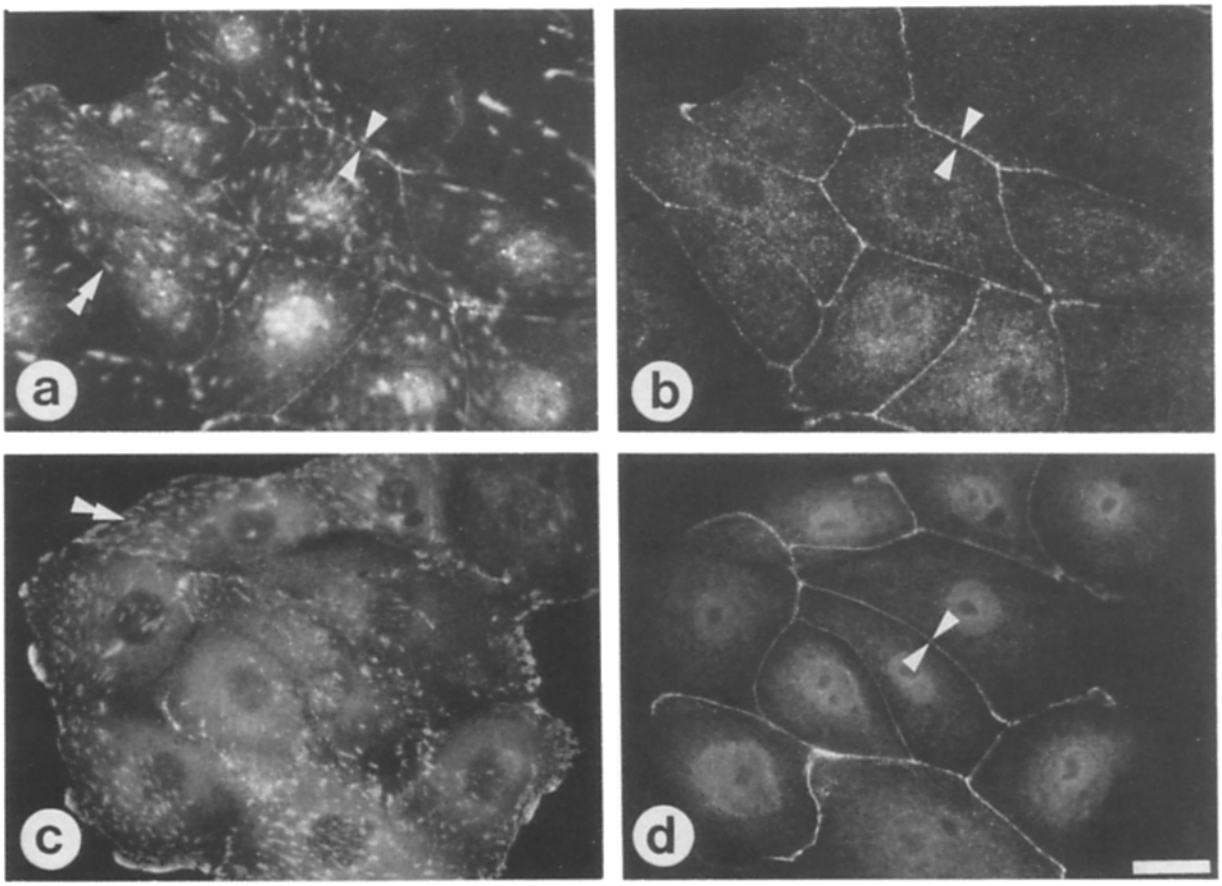

Figure 6. Immunofluorescent localization of talin, vinculin, and uvomorulin in epithelial cells. MDBK cells were double labeled for vinculin (*a*) and uvomorulin (*b*), or for talin (*c*) and uvomorulin (*d*). Vinculin was enriched in both focal adhesions (*double arrowheads* in *a*) and in ZA (between *arrowheads* in *a*) while uvomorulin staining served as an exclusive marker for ZA (between *arrowheads* in *b* and *d*). Talin was found exclusively in focal adhesions (*double arrowheads* in *c*) and was absent from the ZA (between *arrowheads* in *d*). Bar, 20 μm.

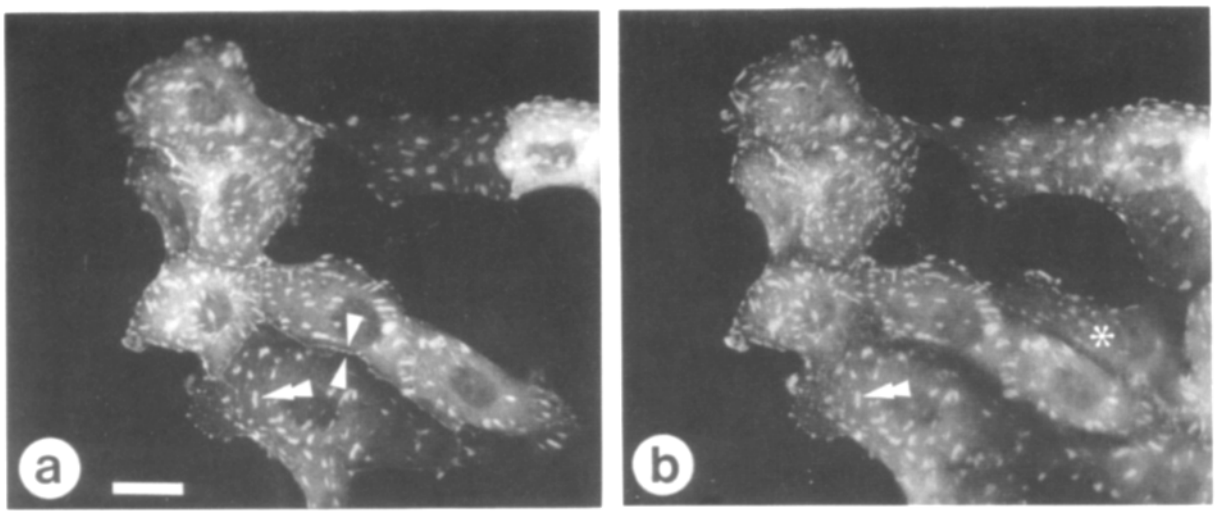

Figure 7. Microinjection of avian smooth muscle vinculin into epithelial cells. MDBK cells were microinjected with purified chicken gizzard vinculin. After a 2-h incubation, the cells were fixed and permeabilized and the microinjected vinculin (*a*) was selectively localized using an mAb that recognizes avian vinculin but not mammalian vinculin. Endogenous talin (*b*) was localized in the same cells using a polyclonal antibody. The microinjected vinculin concentrated in focal adhesions (*double arrowheads* in *a* and *b*) and in ZA (between *arrowheads* in *a*). The localization of talin in focal adhesions (*double arrowheads* in *b*) and its absence from ZA was not affected by vinculin microinjection. The localization of endogenous talin in cells microinjected with vinculin was indistinguishable from cells that had not been microinjected (*asterisk* in *b*). Bar, 20 μm.

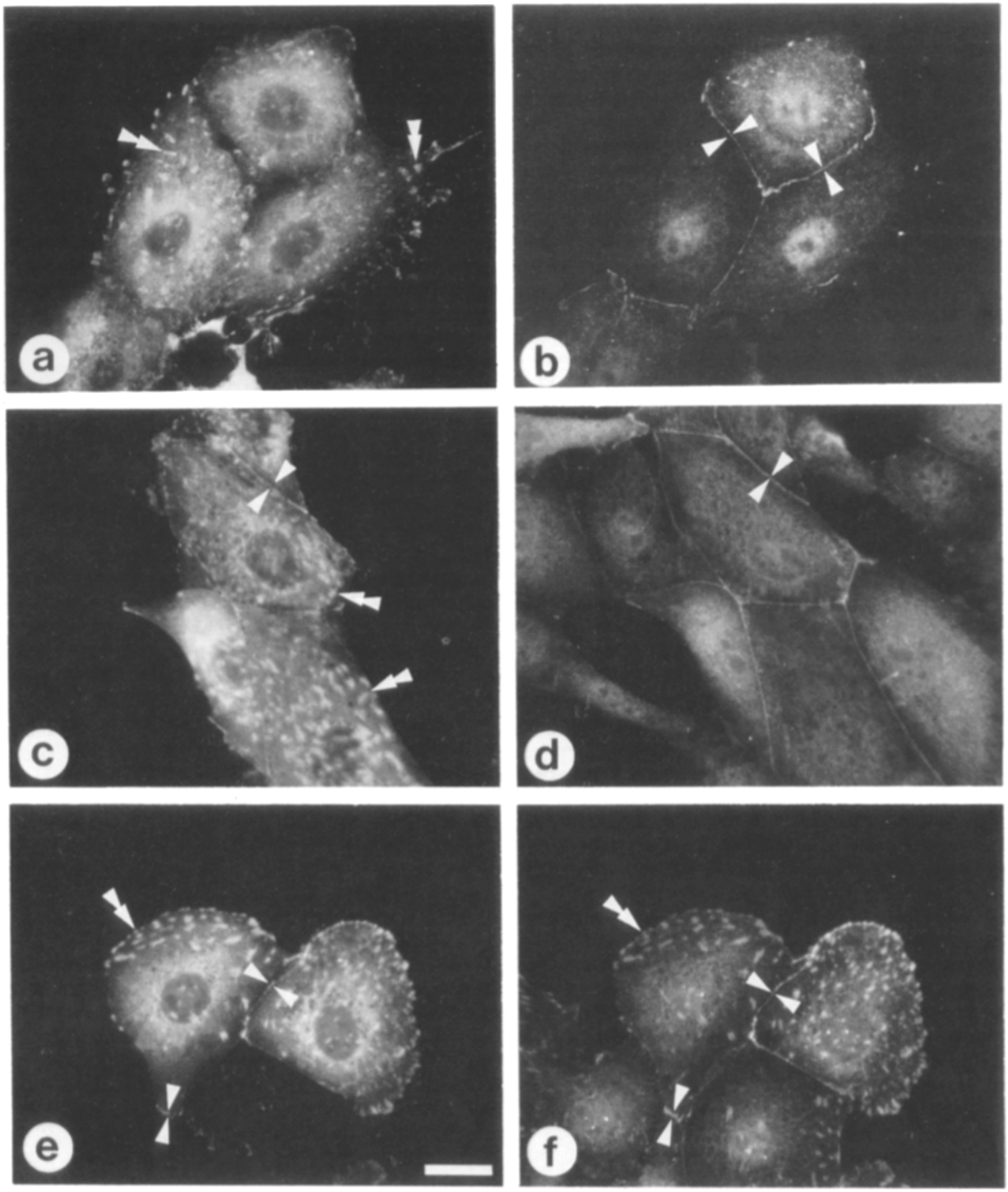

Figure 8. Microinjection of intact talin or the large talin fragment into epithelial cells. MDBK cells were microinjected with intact, unlabeled chicken gizzard talin which was later selectively localized using an avian-specific, talin mAb. Staining for microinjected talin (*a*) compared with uvomorulin in the same cells (*b*) showed that microinjected talin, like the endogenous molecule, localized to focal adhesions (*double arrowheads*) and was excluded from ZA (between *arrowheads*). The large talin fragment however, incorporated into both focal adhesions (*double arrowheads* in *c*) and into ZA (between *arrowheads* in *c*) as identified by uvomorulin staining (*d*). The large talin fragment (*e*), like vinculin (*f*), was enriched in both focal adhesions (*double arrowheads*) and in ZA (between *arrowheads*). Bar, 20 μm.

gle arrowheads). Uvomorulin localization in the same cells confirmed the position of the ZA (between *single arrowheads* in Fig. 8 *d*). The ZA are near the apical surface of the cell while the focal adhesions are associated with the ventral plasma membrane. Since the presence of the large talin fragment in ZA was the unexpected feature of its localization, photographs were taken focusing on the ZA which resulted in the focal adhesions appearing out of focus in these cells.

The localization of microinjected large talin fragment to focal adhesions as well as ZA was also obtained when MDBK were microinjected with unlabeled large talin fragment from chicken, followed by immunolocalization of the injected talin fragment with the chicken specific antitalin mAb (data not shown). We have not detected a functional difference between chicken and human talin or their fragments in the assays that we have performed. MDBK cells containing the fluorescent large talin fragment in focal adhesions (Fig. 8 *e*, *double arrowheads*) and ZA (between *single arrowheads* in Fig. 8 *e*) were immunostained for vinculin (Fig. 8 *f*), which demonstrated that vinculin was present in both of these structures in injected cells as well as noninjected cells. As controls, MDBK cells were microinjected with fluorescein-labeled ovalbumin or rhodamine-labeled BSA. These injected control probes were distributed diffusely throughout the cytoplasm and did not concentrate at ZA (data not shown).

Discussion

In this paper we have explored the behavior of talin domains introduced into live cells by microinjection. The large domain of talin localized to the focal adhesions of both fibroblasts and epithelial cells. Somewhat surprisingly, this talin fragment also accumulated within epithelial cell ZA, sites that normally do not contain talin. When used to overlay Western blots of whole fibroblasts or fractionated chicken gizzard proteins, the large talin fragment, like intact talin (Burridge and Mangeat, 1984), bound to vinculin and metavinculin. Vinculin is prominent in both focal adhesions and ZA and is likely to contribute to the accumulation of the large talin fragment at both of these sites.

The small domain of talin also localized to focal adhesions in fibroblasts, but a substantial cytoplasmic pool was visible. The native state of the small domain of talin may not be well-preserved through cleavage, purification, and fluorescent labeling. Because of high background fluorescence, we were not able to determine whether the small talin fragment localized to focal adhesions or ZA after microinjection into MDBK cells. Nevertheless, these experiments have demonstrated that talin has at least two sites that contribute to its localization to focal adhesions in fibroblasts.

The radioiodinated small talin fragment did not show a consistent interaction with any polypeptide in Western blots of whole fibroblasts or fractionated gizzard. It should be noted that we have not observed specific binding of either of our talin fragments or even uncleaved talin with integrins that are present in the Western blots, and so these assays do not allow us to map the integrin binding site to either of the talin fragments. In previous work, which employed an assay designed to detect low-affinity interactions, it was reported that the large talin fragment bound to integrin (Horwitz et al., 1986). Since we observed the small talin fragment localizing to focal adhesions, this suggests either that the small fragment also has an integrin-binding site, or that this fragment associates with some additional component of focal adhesions.

We were also surprised that the large talin fragment localized to ZA in epithelial cells and thus behaved differently from both the cells' intrinsic talin as well as intact talin introduced by microinjection. Until we obtained these results, we suspected that the normal distribution of talin in epithelial cells was strictly determined by the presence of "receptors" for talin in focal adhesions, and their absence from ZA. The presence of vinculin in ZA seemed to contradict this notion until Bendori et al. (1989) described a cDNA clone which encodes a form of vinculin that does not bind talin. It could be argued that two isoforms of vinculin expressed in the same epithelial cell could account for both the absence of talin-binding sites in ZA and the colocalization of talin and vinculin in focal adhesions. As part of this argument, it would be necessary to invoke some mechanism for segregating the two isoforms of vinculin, such that one went to focal adhesions and the other to ZA. The localization of the large talin fragment in ZA demonstrates that there are talin-binding sites in the ZA. Furthermore, chicken gizzard vinculin microinjected into epithelial cells localized to both focal adhesions and ZA which argues against any mechanism of segregating vinculin isoforms.

We can conceive of at least two explanations that reconcile the absence of talin from ZA and the presence of vinculin and binding sites for the large talin fragment in these structures. One possibility is that the intact talin molecule is excluded from ZA due to steric hindrance such that the potential talin-binding site in ZA (e.g., vinculin) is inaccessible to the intact protein but accessible to the large fragment. Alternatively, there may be a ZA protein that competes with talin for binding to vinculin. This protein may exclude talin from ZA by binding to vinculin with an affinity greater than that of the intact talin molecule, thus displacing talin. However, loss of the small domain may enhance the affinity of the large talin fragment for vinculin such that it accumulates in the ZA. In the future we plan to test these possibilities by comparing vinculin's affinity for intact talin and large talin fragment, and by designing experiments that could identify novel vinculin-binding proteins in epithelial cells.

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