

Accumulation of Talin in Nodes at the Edge of the Lamellipodium and Separate Incorporation into Adhesion Plaques at Focal Contacts in Fibroblasts

Joseph A. DePasquale and Colin S. Izzard

Department of Biological Sciences, State University of New York, Albany, New York 12222

Abstract. The focal contact forms beneath F-actin-rich ribs, or cytoplasmic precursors, present in the lamellipodia of fibroblasts. The basal part of the precursor is retained at the contact as the initial adhesion plaque. We have examined the distribution of talin in the lamellipodia and adhesion plaques of chicken embryo fibroblasts relative to the process of focal contact formation. Motility of single cells was recorded with differential interference contrast or interference reflection microscopy before fixation and fluorescent staining for talin, F-actin, and vinculin. Talin is present along the extreme edge of the lamellipodium, where it is further concentrated into a series of nodes. The nodes of talin are present at the tips of both larger and finer F-actin-rich ribs and at small structural nodes at the

edge of the lamellipodium. We suggest that the talin in the nodes functions, via a cross-linking activity, in the convergence of actin filaments at the membrane during development of the ribs. Talin accumulates *de novo* in the adhesion plaque, independent of that at the tip of the precursor, in response to contact with the substrate. This second accumulation of talin at the focal contact starts before vinculin, consistent with a sequential binding of talin at the membrane and of vinculin to talin. The results imply that talin functions independently at two steps during formation of the focal contact: the development of the F-actin-rich precursor of the contact; and development of the contact-associated adhesion plaque, both involving organization of F-actin at the membrane.

THE focal contact in cultured fibroblasts is a site of close apposition and relatively strong adhesion of the outer surface of the plasma membrane to the substrate (Abercrombie and Dunn, 1975; Izzard and Lochner, 1976, 1980). A patch of amorphous material, the adhesion plaque, is present on the cytoplasmic face of the membrane of the focal contact into which the microfilaments of stress fibers insert (Abercrombie et al., 1970; Heath and Dunn, 1978). The well-characterized cytoplasmic proteins, vinculin, talin and α -actinin, are concentrated at the focal contact and form part of the adhesion plaque (Geiger, 1979; BurrIDGE and Connell, 1983*a,b*; Wehland et al., 1979). An increasing number of other cytoplasmic proteins have also been shown to be concentrated at the focal contact by immunofluorescent staining (reviewed in BurrIDGE et al., 1988). The functional roles of these proteins in the adhesion plaque are being inferred in part from the properties of the proteins and proteolytic fragments in solution, sequence data, and transfection of cells with cDNA clones and fragments (e.g., see BurrIDGE et al., 1988; Otto, 1990). However, it is important to study directly the temporal and spatial distribution of the proteins in the cell, and to determine the sequence of accumulation of the proteins during the initial formation and subsequent maturation of the focal contact and adhesion plaque. This direct information at the cell level will provide a basis for iden-

tifying which properties of the proteins are functionally significant and for understanding how those properties are sequentially regulated during assembly of the focal contact and adhesion plaque.

New focal contacts are formed at the spreading margin of fibroblasts by lamellipodia or microspikes, which extend from the cell margin free from the substrate (Izzard and Lochner, 1980). Individual focal contacts form beneath linear cytoplasmic precursors. The linear precursors consist of fibers which appear as "ribs" within the structure of the motile lamellipodium, or the cores of microspikes extending proximally through the lamellipodium, or the cylindrical microspikes themselves (Izzard and Lochner, 1980; Izzard, 1988). The three forms of the precursor of the focal contact are interchangeable with one another. Each stains intensely with NBD-phalloidin, and therefore contains a high concentration of F-actin relative to the adjacent lamellipodium (DePasquale and Izzard, 1987).

The initial adhesion plaque is derived structurally from the part of the linear precursor beneath which the focal contact forms. It is rich in F-actin from the beginning, consistent with the interpretation that F-actin of the motile precursor is transferred to the initial adhesion plaque (Izzard and Lochner, 1980; DePasquale and Izzard, 1987). We have shown previously that vinculin is not concentrated in the motile

precursor, but accumulates progressively in the adhesion plaque after the focal contact forms (DePasquale and Izzard, 1987). The observations on the timing of incorporation of vinculin into the plaque are consistent with the general view that vinculin is not concentrated in lamellipodia or ruffles (Geiger, 1979).

In contrast to vinculin, several proteins of the mature adhesion plaque are reported to be concentrated in lamellipodia and marginal ruffles, e.g., α -actinin (Lazarides, 1976), talin (Burrige and Connell, 1983*a,b*), and fimbrin (Bretschner and Weber, 1980). Therefore, in view of the structural continuity between the unattached motile cytoplasmic precursor of the focal contact and the adherent adhesion plaque, incorporation of any one of these proteins into the adhesion plaque could begin in the lamellipodium at the level of the precursor prior to contact with the substrate. In this context, we have examined by indirect immunofluorescence the detailed distribution of talin in nonadherent lamellipodia and in adhesion plaques associated with focal contacts of known age. Video records of the live cell were made before fixation for immunofluorescent staining using interference reflection microscopy (IRM)¹ to identify motile lamellipodia and new focal contacts, and differential interference contrast (DIC) to identify motile precursors and new adhesion plaques. The distribution of talin was correlated with that of F-actin or vinculin by parallel NBD-phalloidin or immunofluorescent staining, respectively. The results show that talin accumulates in two locations that are spatially and temporally distinct, and independent of one another. The first is a novel location at the tips of the F-actin-rich linear precursors of the focal contact; the second develops at the adhesion plaque after the focal contact forms. Preliminary discussion of parts of this work has been presented (Izzard, 1988).

Materials and Methods

Cell Culture

Cultures of chicken embryo fibroblasts (CEFs) were prepared from trypsinized 9–11-d-old embryos and maintained in DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS (Flow Laboratories, Inc., McLean, VA), nonessential amino acids and 50 U/ml penicillin and 50 μ g/ml streptomycin (Gibco Laboratories). Chick heart fibroblasts were prepared from trypsinized ventricles. For microscopy, cells were seeded on coverslips and used within 24 h. Coverslips were inverted on spacers and sealed along two sides leaving two ends open as a simple perfusion chamber. The preparations were maintained at 37°C on the microscope with an air-curtain incubator (Sage Instruments Div., Orian Research Inc., Cambridge, MA). The motile history of an individual cell was recorded, and the cell fixed and processed for fluorescent staining by perfusion through the chamber while on the microscope.

Light Microscopy and Video Recording

DIC, IRM, and fluorescence microscopy were performed as previously described (DePasquale and Izzard, 1987) using a Universal Microscope and 100/1.25 planachromat oil-immersion objective lens (Carl Zeiss, Inc., Thornwood, NY). The DIC and IRM images of live cells were recorded with a Newvicon video camera (67M; Dage-MTI, Inc., Michigan City, IN) and the associated fluorescent staining after fixation was recorded with an ISIT video camera (66 ISIT; Dage-MTI, Inc.). Images from the ISIT camera were noise-reduced in real-time by recursive frame-averaging using an Intellect 100 image processor (MCI Quantel, Micro Consultants, Inc., Palo Alto, CA). The ISIT camera was operated mostly in the automatic mode.

1. *Abbreviations used in this paper:* CEF, chicken embryo fibroblast; DIC, differential interference contrast; IRM, interference reflection microscopy.

No additional localized fluorescence could be detected by increasing the gain of the camera in the manual mode, or by digital enhancement using the integration function of the image processor. The video images were mixed with a time-base, providing video-field number (model 346; Thalner Electronics Lab., Inc., Ann Arbor, MI), and recorded with a 1/2-inch VCR (model 8050; Panasonic Co., Secaucus, NJ). The motile history of individual live cells and fixation were recorded at a time-lapse rate of every twelfth video-field, and subsequent processing and final fluorescent images at the normal video-frame rate. The Newvicon and ISIT cameras introduced no differences in magnification or geometric distortion, and the respective images could be compared directly. Conventional 35 mm photomicrographs of IRM and fluorescence images were taken on a Photomicroscope II (Carl Zeiss, Inc.).

Fixation and Fluorescent Staining

Cells in culture medium were perfused at 37°C with 3.7% paraformaldehyde in the low calcium, buffered salt solution of Small (1981), pH 7.2, and fixed for 5 min. Subsequent steps were at 23°C and used this buffer. The fixed cells were permeabilized with 0.5% Triton-X 100 for 20 min, treated with 0.1 M glycine for 10 min, and washed with buffer. Some cells were incubated with NBD-phalloidin (Molecular Probes, Inc., Eugene, OR) at 50 ng/100 μ l for 20 min, washed and then stained for talin; other cells were stained for talin and vinculin. Immunofluorescent staining for talin used a rabbit antitalin serum at 1:250 dilution provided by Dr. Keith Burrige (University of North Carolina, Chapel Hill); that for vinculin used a guinea pig antivinculin serum at 1:25 dilution provided by Dr. Irwin Singer (Merck, Sharp and Dohme Research Laboratories, Rahway, NJ) or a mouse antivinculin mAb at 1:100 dilution (Miles Scientific, Naperville, IL). Secondary antibodies were TRITC-goat anti-rabbit IgG (affinity pure), FITC-goat anti-guinea pig IgG (both from Cappel Laboratories, Malvern, PA), and FITC-goat anti-mouse Fab (affinity pure) (Miles Scientific) each used at 1:50 dilution. After final washing, cells were observed in buffer for all optical methods.

Results

Distribution of Talin at the Leading Edge of CEF

Talin was shown previously to be concentrated in three primary locations in cultured fibroblasts: in the adhesion plaques associated with focal contacts; to a lesser and variable extent along F-actin-rich stress fibers; and in the actin-rich marginal ruffles at the edge of the leading lamella (Burrige and Connell, 1983*a,b*). The three distributions of talin relative to that of F-actin are illustrated in Fig. 1, and compared with an IRM image of the same cell. We have found consistently with respect to the marginal ruffles, or more strictly lamellipodia, that talin is concentrated at the extreme edge, not over the full breadth, of the F-actin-rich lamellipodium (Fig. 1, *A–C*). In addition, the talin is further concentrated into a series of more brightly staining nodes at the edge of the lamellipodium (Fig. 1 *B*). The nodes of talin do not represent small sites of substrate adhesion. When the motile activity of the cell was recorded using IRM before fixation and staining, the nodes of talin were shown to be present at the distal edge of freely motile, unattached lamellipodia (see below).

Distribution of Talin Relative to Structures within the Lamellipodium

Lamellipodia contain distinct F-actin-rich rib-like structures that extend radially through the full width of the lamellipodium (Fig. 1, *A* and *C*). The ribs move with the lamellipodium as a whole, and also can move transversely within the lamellipodium. This motion was recorded using DIC optics before fixation and staining for talin and F-actin. Talin was found to be concentrated without exception at the tip of

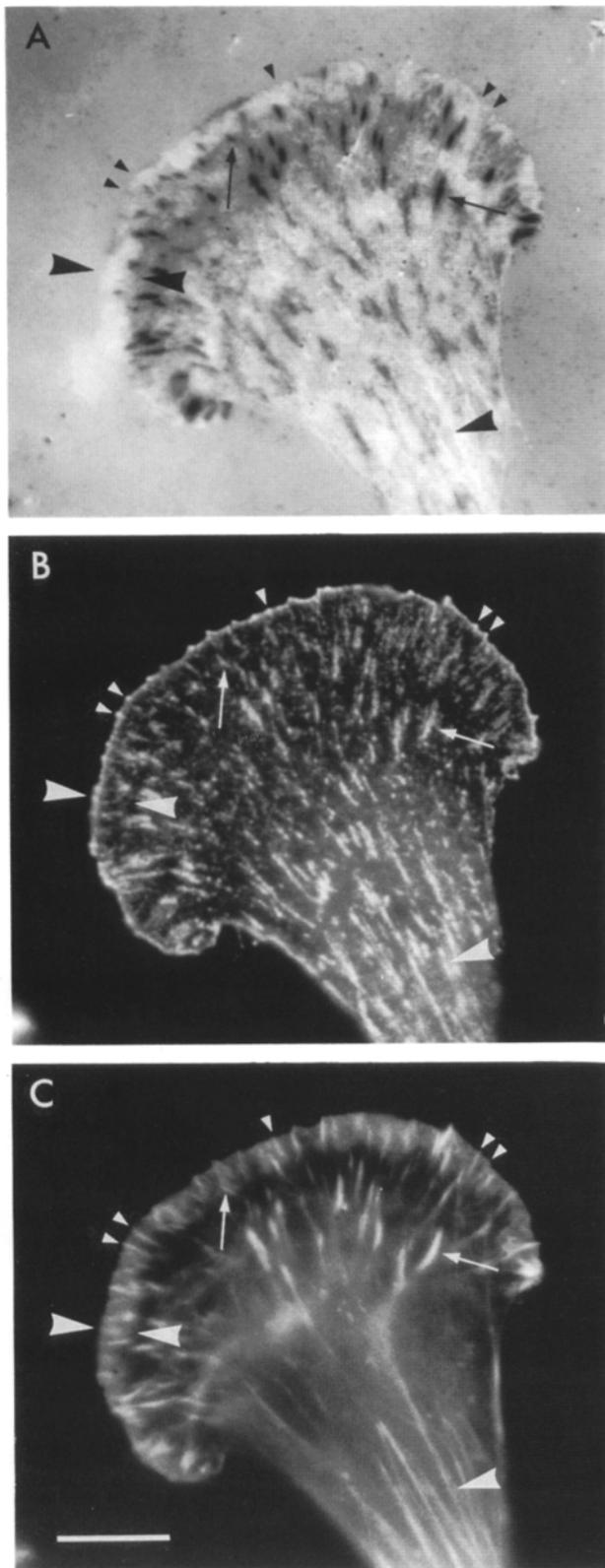


Figure 1. Three primary locations of talin in a typical chick heart fibroblast. Photomicrographs of the same cell after fixation and fluorescent staining: (A) IRM image; (B) talin distribution, indirect immunofluorescence; (C) F-actin, NBD-phalloidin staining. Talin is concentrated, together with F-actin, over each focal contact, large or small, in the broad leading lamella (*matching arrows*). Talin is present in segments along the length of F-actin stress fibers in the cell body in the absence of focal contacts (*matching*

each motile F-actin-rich rib (Fig. 2, A, C, and D, *matching arrowheads*). The staining was concentrated typically over the distal 1.0–1.5 μm of the rib. There was either no staining for talin along the length of the F-actin-rich ribs, or only weak staining along the distal third of the rib, compared to that in the adjacent lamellipodium (Fig. 2, C and D). Where the ribs extended 2 μm or more beyond the edge of the lamellipodium as microspikes, and in microspikes extending directly from the cell surface, talin was preferentially concentrated at the tip, not along the full length of the microspike (Fig. 3, A, B, and D).

Other nodes of talin were present at the edge of the lamellipodium between the major F-actin ribs. Some were present at the distal ends of structurally less prominent ribs that stained less intensely for F-actin (Fig. 2, A, C, and D, *matching double arrowheads*). Other nodes of talin, as discrete spots of staining (Fig. 3 B, *top arrowheads*), or local increases in intensity of the staining along the edge of the lamellipodium (Fig. 3 B, *bottom arrowheads*), coincided with structural nodes at the edge of the lamellipodium in parallel DIC images (Fig. 3, B and D, *matching arrowheads*). In these cases, the lamellipodium behind the nodes did not contain clear F-actin-rich, structural ribs. Instead, the F-actin staining sometimes showed a beaded, lattice-type of pattern (Fig. 3 A, *opposite bracket*). By comparison with previous ultrastructural studies (Small, 1981), we interpret this staining pattern as indicative of the presence of an orthogonal lattice of fine bundles of actin filaments that stain more intensely at the cross-over points.

An alternative explanation for the increased fluorescent staining for talin at the edge of the lamellipodium and in the nodes, namely that the increased fluorescent intensity results simply from a uniform association of talin with the plasma membrane and curvature of the membrane through the depth-of-focus of the microscope, is not supported by the following facts: (a) The fluorescent staining for talin is discontinuous along the edge of the lamellipodium (see Figs. 2 D, 3 B, 5 D); (b) the fluorescence is absent along the length of microspikes (Fig. 3 B); and (c) the increased fluorescent intensity extends 0.5–1.0 μm behind the edge of the lamellipodium (Fig. 2 D), a distance much greater than the radius of curvature of the membrane at the edge of the lamellipodium, which is only 0.16 μm thick (Abercrombie et al., 1971).

Relationship between Talin in the Nodes and in Adhesion Plaques

The focal contact forms beneath the actin-rich ribs in the lamellipodium which function, therefore, as structural precursors of the contact. The basal part of the rib segregates from the distal part and is retained at the new contact as

large arrowheads). A lamellipodium is present across the convex margin of the leading lamella. It generates a reflection of higher intensity than the adjacent coverslip in the IRM image (A) and stains more intensely for F-actin than the leading lamella (C). Talin is concentrated along the edge of the lamellipodium (for example, compare areas between matching opposing arrowheads in A–C). Talin is further concentrated into brightly stained nodes at the tips of F-actin-rich ribs in the lamellipodium (*matching small arrowheads* in A–C). Bar, 10 μm .

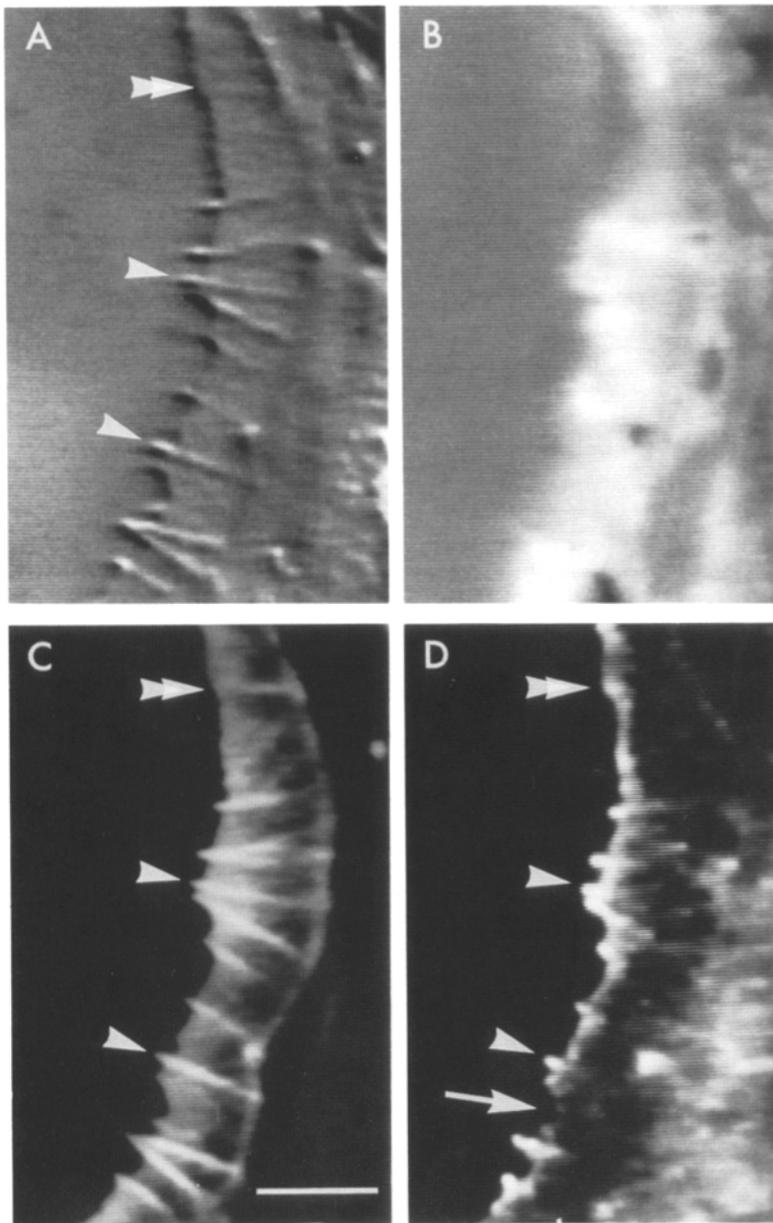


Figure 2. Distribution of talin in a lamellipodium of a CEF. The motility of the lamellipodium was recorded before fixation using DIC optics. Videomicrographs: (A) DIC image and (B) IRM image immediately after fixation; (C and D) Fluorescence images after permeabilization and staining for F-actin with NBD-phalloidin (C) and for talin with rhodamine-labeled second antibody (D). Well-developed structural ribs extended from the edge to the base of the lamellipodium and stained intensely for F-actin (A and C, *matching arrowheads*). The lamellipodium and contained ribs were motile in the live cell. After fixation, neither showed contact with the substrate in the IRM image (B). Staining for talin (D) occurred along the edge of the lamellipodium, and in an irregular patchy distribution behind the base of the lamellipodium. There was little staining over the width of the lamellipodium. Along the edge of the lamellipodium talin was concentrated at the tip of each F-actin-rich rib (A, C, and D, *matching arrowheads*), and in nodes at the tips of less well developed ribs (A, C, and D, *matching double arrowheads*). Staining was discontinuous at points along the edge of the lamellipodium (D, *arrow*). Bar, 5 μm .

the initial adhesion plaque (Izzard and Lochner, 1980; DePasquale and Izzard, 1987). By recording this process with DIC optics before staining for talin, we could show that talin is present at the tip of the precursor while a new accumulation of talin develops at the adhesion plaque. For example, the transition from a motile precursor, consisting of a microspike and its core extending proximally into the cell margin, to a precursor apparently attached along its basal part is followed by arrow 1 in Fig. 4, A–C. In the fixed cell the precursor stained throughout its length for F-actin (Fig. 4 E) and a focal contact was present under the basal part of the precursor (Fig. 4 D). Talin was concentrated at the tip of the precursor and along the adherent basal part of the precursor, or new adhesion plaque (Fig. 4 F, *arrowhead* and *arrow 1*, respectively). Arrow 2 follows the segregation of a precursor into an adhesion plaque associated with a focal contact (Fig.

4, A–D) and a distal motile part (Fig. 4 C, *arrowhead*). Both structures stained for F-actin. Talin was present at the new plaque and the tip of the original precursor (Fig. 4, E and F, *matching arrow 2* and *arrowheads*, respectively). A third example is shown in the same cell (Fig. 4, *arrow 3* and *associated arrowhead*). These observations imply that talin in the adhesion plaque is not derived from that at the tip of the precursor, but accumulates independently in response to contact with the substrate.

Accumulation of Talin and Vinculin at New Focal Contacts

The motility of lamellipodia and formation of new focal contacts were recorded using IRM, and then the cells were fixed and stained for talin and vinculin. These records demon-

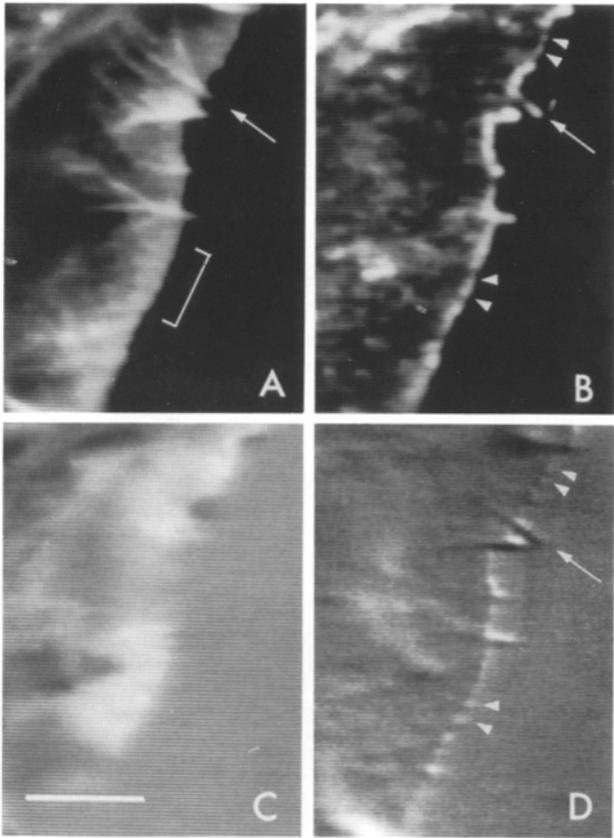


Figure 3. Distribution of talin in a recently developed lamellipodium of a CEF. The lamellipodium was present at the advancing margin of a leading lamella. Videomicrographs: (A and B) Fluorescence images after staining for F-actin with NBD-phalloidin (A) and for talin with rhodamine-labeled second antibody (B); (C) IRM image and (D) DIC image immediately after fixation. Video-DIC records of the live cell showed that the lamellipodium was motile and the lower-half extending at the time of fixation. No contacts were present between the lamellipodium and substrate in the IRM image (C). Talin was concentrated preferentially at the tip, not along, the larger microspikes (A, B, and D, *matching arrows*). Talin along the edge of the lamellipodium was concentrated into discontinuous nodes (B, *upper arrowheads*) or nodes of more intense staining (B, *lower arrowheads*) that corresponded to small structural nodes in the DIC image (D, *matching arrowheads*). Note the beaded pattern of F-actin staining within the lamellipodium (A, *opposite bracket*) that lies behind the nodes marked by the lower arrowheads (B and D). Bar, 5 μm .

strated directly that the nodes of talin at the edge of the lamellipodium did not correspond to small adhesion sites (Fig. 5, A, B, and D), nor contain vinculin (Fig. 5, C and D). The age of each new focal contact at fixation was determined from the video records, and correlated with the degree of staining for talin and vinculin at the contact (Fig. 5). The results are summarized in Table I. Except for two, all the new focal contacts, including the youngest group, stained for talin. The talin staining increased in a punctate manner rather than uniformly in intensity throughout the contact area. In $\sim 20\%$ of the contacts, talin was present as a spot smaller than the contact. This condition could last up to 180 s (Table I; *Tn+*). The staining for talin appears to pro-

gress to irregular patches partially covering the contact area and then to a more intense staining of the whole contact area. The more intense staining appeared early, in <30 s in some contacts, and began to peak in contacts >61 s old (Table I, *Tn+++*). In contrast, $\sim 18\%$ of the new contacts, ranging up to 180 s old, were unstained for vinculin (Table I, *Vn-*); strong staining for vinculin occurred only in contacts >150 s old (Table I, *Vn+++*). Table II compares the staining for the two proteins in individual contacts. All contacts lacking vinculin, except for one, stained for talin (Table II, *top row*; Fig. 5, B, C, and D, *middle arrow*). The reverse situation was rare with only one contact staining weakly for vinculin in the absence of talin (Table II, *first column*). Of the contacts unstained or weakly stained for vinculin (Table II, *Vn-* and *Vn+*), $\sim 30\%$ were intensely stained for talin (*Tn+++*). The analysis shows that the accumulation of talin at the new contact begins before vinculin, in some cases to significant levels, and continues ahead of vinculin, at least by the qualitative criteria used to assess accumulation.

Discussion

In this study we have examined in detail the spatial distribution of talin in the lamellipodium of avian fibroblasts, and evaluated the relationship of the lamellipodial talin to that in the adhesion plaques of new focal contacts. We found a novel feature of the distribution of the protein in the lamellipodium. Talin is present along the extreme edge, not across the whole breadth of the lamellipodium, and it is further concentrated into nodes at the edge of the lamellipodium. A node of talin is present at the tip of every F-actin-rich rib in the lamellipodium, and thus at the tip of the structures that function as precursors of the focal contact (DePasquale and Izzard, 1987; Izzard, 1988). However, we found no evidence that talin in the lamellipodium gives rise to that in the adhesion plaque. Instead, our analyses showed that incorporation of talin into the adhesion plaque occurs de novo as the focal contact forms and therefore in response to contact of the cell surface with the substrate.

The accumulation of talin along the edge of the lamellipodium is clearly independent of contact with the substrate. This feature is unusual. In most cases talin accumulates adjacent to segments of the plasma membrane that are adherent to the substrate as focal contacts or associated with fibronectin fibrils in cultured cells (Burrige and Connell, 1983a,b), or apposed to the extracellular matrix in tissues, as in the smooth muscle dense plaque (Geiger et al., 1985), the skeletal muscle myotendinous junction (Tidball et al., 1986), and other epithelial and mesenchymal cells where they are in contact with the basal lamina and connective tissue (Drenckhahn et al., 1988). These submembrane accumulations of talin at sites of matrix interaction are consistent with the ability of talin to bind to the integrins (Horwitz et al., 1986; Buck and Horwitz, 1987), and with the parallel accumulation of talin and integrins in cultured cells at focal contacts (Fath et al., 1989) and extracellular matrix contacts (Burrige and Connell, 1983b; Chen et al., 1985; Mueller et al., 1988). Under experimental conditions, talin and integrin collect together along the site of contact between fibroblasts in suspension and beads coated with fibronectin or antibodies against the integrin β_1 -subunit, but not in the case of beads

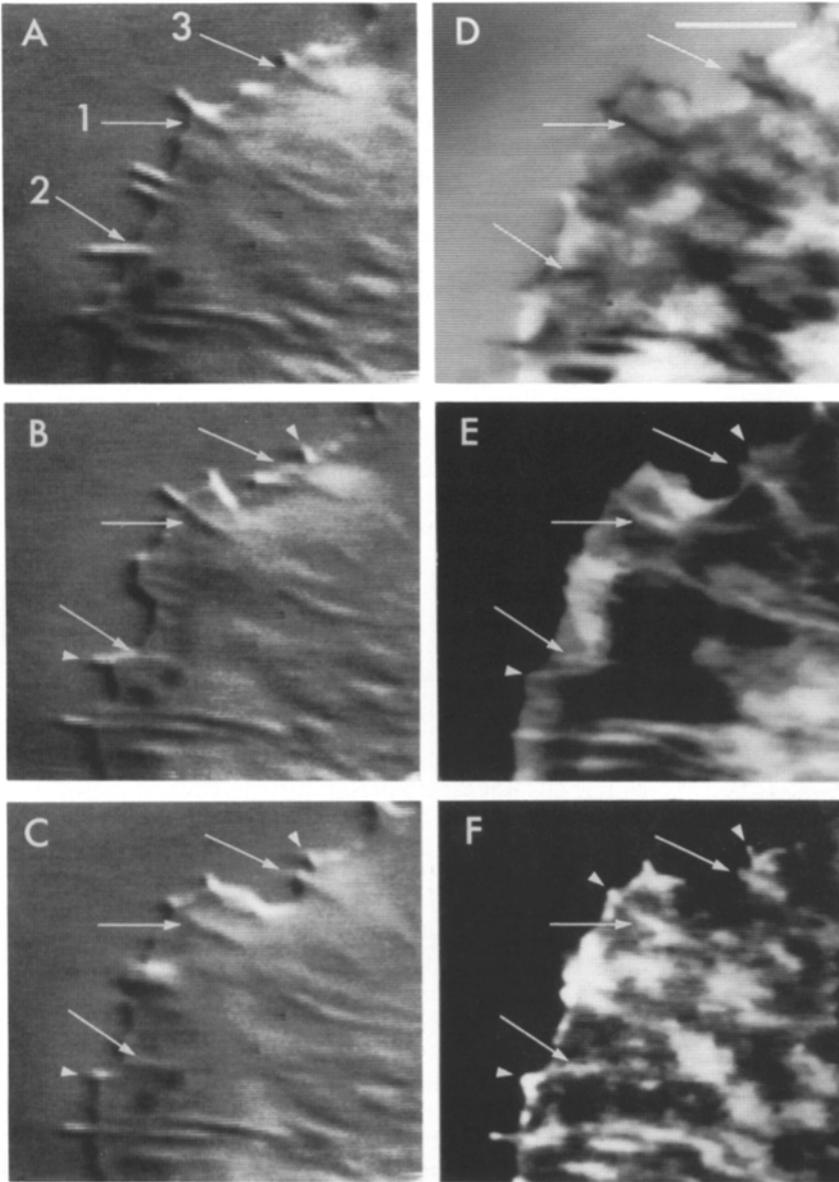


Figure 4. Distribution of talin and F-actin in new adhesion plaques in CEF. The motility of cytoplasmic precursors, and the attachment of the basal part of the precursor to form an adhesion plaque, were recorded in the live cell by video-DIC before fixation and staining. The presence of focal contacts beneath adhesion plaques was confirmed in the fixed cell by IRM. Videomicrographs: (A–C) DIC images of live cell, time before fixation (min/s), A (1:34), B (0:36), C (0:01); (D) IRM image after fixation and permeabilization; (E and F) Fluorescence images after staining for F-actin with NBD-phalloidin (E) and for talin with rhodamine-labeled second antibody (F). The formation of three separate adhesion plaques is followed by the numbered arrows in A and matching arrows in B–F. (arrow 1) The precursor consisted of a microspike and its core extending into the cell margin (A). Both were motile. The microspike elongated distally and the core proximally, while the edge of the lamellipodium advanced up the core of the microspike (B). The core rotated anti-clockwise and, 14 s before fixation, thickened slightly, became stationary, and retained the form shown just before fixation in C. A focal contact was present beneath the apparently adherent part of the precursor (D). F-actin was present throughout the length of the precursor (E). However, talin was concentrated at the tip of the original precursor (F, arrowhead) and along the segment of the precursor overlying the focal contact (F, arrow). (arrow 2) The core of the microspike in A elongated into the motile cell margin and became stationary forming a typical adhesion plaque (B, arrowhead). The two components were present up to fixation, the distal part having moved further away from the stationary plaque (C, arrowhead and arrow). A focal contact was present beneath the stationary plaque (D). Both structures stained for F-actin (E). Talin was concentrated over the focal contact in the associated adhesion plaque, and at the tip of the distal part of the original precursor (F, arrow and arrowhead). (arrow 3) The basal part of this precursor had become stationary before the time in A. The distal part segregated and moved laterally away from the basal part (B, arrowhead and arrow). Both parts were retained up to fixation (C). A focal contact was present beneath the stationary plaque (D). Both structures stained for F-actin (E). Talin was concentrated in the plaque over the focal contact and in the segregated distal part of the original precursor (F, arrow and arrowhead). Note: The nodes of talin at the tips of the two precursors followed by arrows 1 and 3 (F, associated arrowheads) appear to be in contact with the substrate in the IRM image (D). However, the DIC images of the live cell showed that in each case the distal part of the continuous precursor (arrow 1) and of the segregated precursor (arrowhead associated with arrow 3) were motile up to fixation, and therefore not adherent to the substrate. We have observed from continuous IRM records that an apparent contact of the tips of precursors and microspikes with the substrate can develop during permeabilization or subsequent staining steps. For this reason, it is important to use additional information from DIC or IRM images of the live cell to evaluate the correct contact status of these structures in IRM images of fixed and permeabilized cells. Bar, 5 μm .

tin (E). Talin was concentrated over the focal contact in the associated adhesion plaque, and at the tip of the distal part of the original precursor (F, arrow and arrowhead). (arrow 3) The basal part of this precursor had become stationary before the time in A. The distal part segregated and moved laterally away from the basal part (B, arrowhead and arrow). Both parts were retained up to fixation (C). A focal contact was present beneath the stationary plaque (D). Both structures stained for F-actin (E). Talin was concentrated in the plaque over the focal contact and in the segregated distal part of the original precursor (F, arrow and arrowhead). Note: The nodes of talin at the tips of the two precursors followed by arrows 1 and 3 (F, associated arrowheads) appear to be in contact with the substrate in the IRM image (D). However, the DIC images of the live cell showed that in each case the distal part of the continuous precursor (arrow 1) and of the segregated precursor (arrowhead associated with arrow 3) were motile up to fixation, and therefore not adherent to the substrate. We have observed from continuous IRM records that an apparent contact of the tips of precursors and microspikes with the substrate can develop during permeabilization or subsequent staining steps. For this reason, it is important to use additional information from DIC or IRM images of the live cell to evaluate the correct contact status of these structures in IRM images of fixed and permeabilized cells. Bar, 5 μm .

coated with Con A (Mueller et al., 1989). Talin also accumulates adjacent to antibody-induced caps of integrin in lymphocytes, if the cells are treated with phorbol ester tumor promoters (Burn et al., 1988). These experimental examples demonstrate clearly that a submembrane concentration of talin can develop in response to the binding of an integrin to an immobilized matrix ligand, or to the external cross-

linking of the integrin, and that the induced accumulation of the talin can be metabolically regulated. In preliminary studies, we have found that the β_1 -subunit of the avian integrin is also concentrated in nodes along the edge of the lamellipodium and at the tips of the F-actin-rich ribs in the lamellipodium (Memmo, L. M., and C. S. Izzard, 1989. *J. Cell Biol.* 109:319a, and our unpublished results). However,

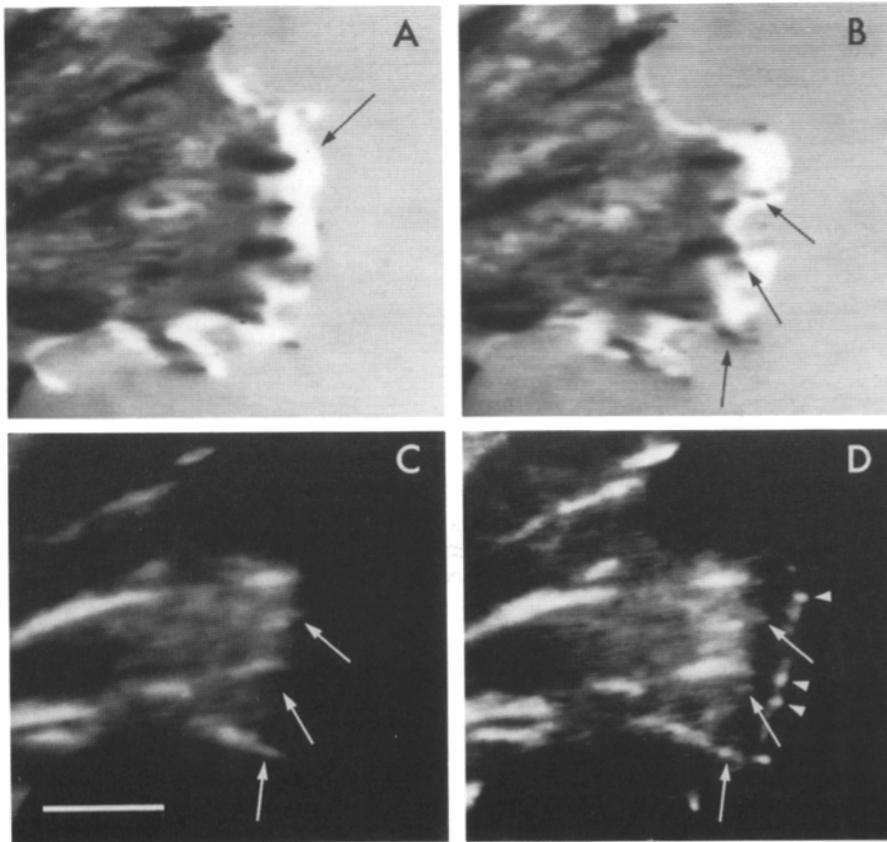


Figure 5. Accumulation of talin and vinculin at new focal contacts in CEF. The motility of lamellipodia and formation of focal contacts in live cells was recorded by video-IRM up to fixation for staining for talin and vinculin. The age of individual contacts at fixation was determined from the records. Videomicrographs: (A and B) IRM images at 36 and 1 s, respectively before fixation; (C and D) Fluorescence images after staining for vinculin with fluorescein-labeled second antibody (C) and for talin with rhodamine-labeled second antibody (D). (A) A motile lamellipodium (arrow), generating a high-intensity reflection, extended ahead of the large focal contacts and close contact beneath the leading lamella. (A to B) Three new focal contacts formed beneath the motile lamellipodium (B, arrows). Talin was associated with each new contact (D, arrows), but the staining was less intense than in the larger preexisting contacts. Vinculin was associated with the upper and lower, but not with the middle contact (C, arrows). Note, talin is also concentrated in nodes along the edge of the lamellipodium (D, arrowheads), where the lamellipodium was not in contact with the substrate in the live cell immediately before fixation (B). The talin nodes (D) did not stain for vinculin (C). Bar, 5 μm .

there is no evidence for an accumulation of fibronectin or other matrix protein at the edge of the lamellipodium, and one cannot account for the distribution of talin at the edge in terms of the effects of external ligands on the integrins. Given the dynamic nature of the lamellipodium, an accumulation of matrix proteins at the edge of the structure is un-

likely. In addition, talin is apparently lost from the lamellipodia of CEF treated with tumor-promoting phorbol esters (Beckerle, 1990), in contrast to the effect of the esters on the induced accumulation of talin in lymphocytes (Burn et al., 1988). Currently, we are examining the extent to which integrin and talin codistribute in the lamellipodium, and the se-

Table I. Staining of New Focal Contacts for Talin and Vinculin (No. of Contacts)

	Age of contacts (s)										Total new contacts
	0-30	31-60	61-90	91-120	121-150	151-180	181-210	211-240	241-270	271-300	
New contacts	9	11	12	15	12	16	4	4	2	2	87
Talin											
Tn-	0	0	2	0	0	0	0	0	0	0	2
Tn+	3	4	2	6	2	3	0	0	0	0	20
Tn++	3	5	2	7	6	4	3	1	1	0	32
Tn+++	3	2	6	2	4	9	1	3	1	2	33
Vinculin											
Vn-	5	2	2	4	2	1	0	0	0	0	16
Vn+	4	8	6	9	5	9	2	1	1	1	46
Vn++	0	1	4	2	5	4	0	0	0	0	16
Vn+++	0	0	0	0	0	2	2	3	1	1	9

The motility of individual CEF was recorded using IRM, and the age of all the new focal contacts determined at the time of fixation. Level of talin staining: Tn-, contact unstained; Tn+, spot smaller than contact; Tn++, irregular patches partially covering the contact area; Tn+++, more intense staining of whole contact area. Level of vinculin staining: Vn-, contact unstained; Vn+, contact very weakly stained in spots; Vn++, contact area weakly and uniformly stained; Vn+++, contact area uniformly and strongly stained (see DePasquale and Izzard, 1987).

Table II. Comparison of Staining for Talin and Vinculin in Individual Focal Contacts (No. of Contacts)

	Talin				Totals for vinculin
	Tn-	Tn+	Tn++	Tn+++	
Vinculin					
Vn-	1	9	3	3	16
Vn+	1	11	19	15	46
Vn++	0	0	7	9	16
Vn+++	0	1	1	7	9
Totals for talin	2	21	30	34	87

The level of staining for talin and vinculin in the focal contacts in Table I is compared in terms of the number of individual contacts showing each possible combination of staining.

quence of incorporation of the two proteins into the focal contact.

The function of talin at the edge of the lamellipodium is unknown. The accumulation of the protein occurs where many of the constituent actin filaments terminate end-on at the plasma membrane. The concentration of talin into nodes parallels the density of actin filaments at the membrane. For example, at the ultrastructural level, filaments grouped into small and large bundles, and many of those forming a less dense orthogonal lattice between the bundles, terminate at the edge of the lamellipodium in patches of material derived from the plasma membrane (Höglund et al., 1980; Small, 1981; Small et al., 1982). This feature of the talin distribution in the lamellipodium is similar to that at the focal contact and other matrix adhesion sites where actin filaments also are grouped end-on at the membrane (reviewed in Burridge et al., 1988). The similarity implies a common function for talin that is not specifically related to adhesion. For example, talin forms dimers in solution at higher protein concentrations, and it has been suggested that dimers of talin cross-link integrins or vinculin and stabilize the focal contact (Molony et al., 1987). We discussed previously (Izzard, 1988) evidence that the F-actin-rich ribs develop in the lamellipodium via two processes: the convergence of the ends of the filaments at the membrane to form structural nodes and associated small oblique fibers; and the inward coalescence of the actin filaments to form the radial ribs. The talin concentrated in the structural nodes (Fig. 3, B and D) and at the tips of the finer and larger F-actin-rich ribs (Fig. 2, C and D) could function in the convergence process through a cross-linking activity at the edge of the lamellipodium, especially since we now find the integrin β_1 -subunit at the tips of the F-actin ribs (Memmo, L. M., and C. S. Izzard, 1989. *J. Cell Biol.* 109:319a, and unpublished results).

How a cross-linking activity by talin in the plane of the membrane could be coupled to the ends of the actin filaments and thus convergence at the edge of the lamellipodium, is unclear. A similar question of the coupling of actin filaments to the membrane has existed for the focal contact (Burridge et al., 1988), because definitive evidence for the binding of actin to vinculin (Otto, 1990), talin (Burridge and Molony, 1990), or integrin has not been readily available. Two recent reports are potentially significant to the question. Alpha-actinin was shown to bind strongly to integrin (Otey et al., 1990), and thus could couple actin filaments indirectly via

integrin to a cross-linking activity of talin dimers during the convergence process. Also, evidence has been presented recently for a binding of talin to F-actin in solution as a result of changes in the method used to isolate talin (Muguruma et al., 1990). This binding could provide an alternate direct coupling between the convergence of actin filaments and a cross-linking activity of talin dimers.

By analyzing the formation of the adhesion plaque from the F-actin-rich precursor of the focal contact, we have shown that talin accumulates de novo in the adhesion plaque independent of the talin at the tip of the precursor (Fig. 4). The accumulation occurs rapidly in response to contact with the substrate and, therefore, must involve different initial regulatory steps from those at the tip of the precursor, even though talin may perform similar functions at each site. Vinculin also accumulates de novo in the new adhesion plaque (DePasquale and Izzard, 1987). By comparing talin and vinculin staining at focal contacts of known age (Table I and II), we showed that talin can accumulate to significant levels at the new contact before vinculin is detectable. Since a polyclonal antiserum and an mAb were used for the vinculin staining with no difference in results, it is unlikely that only a late accumulating isoform of vinculin was detected at the focal contact. The simplest explanation of these results is that the initial accumulation of the two proteins occurs by a sequential binding of talin to the membrane, presumably via integrin β -subunits, and then of vinculin to talin. The sequence is consistent with the strong binding of vinculin to talin in vitro (Burridge and Mangeat, 1984), and with the ability of integrin, talin, and vinculin to form complexes in solution (Horwitz et al., 1986). In addition, a talin-dependent incorporation of vinculin into focal contacts has been demonstrated in vivo through transfection experiments. Avian vinculin expressed in COS cells from cDNA lacking the sequence for the COOH-terminal tail of the molecule incorporated into contacts, whereas that from cDNA lacking the sequence for a talin-binding domain in the globular head as well as the sequence for the tail of the molecule failed to incorporate into the contacts (Bendori et al., 1989; Jones et al., 1989). Nevertheless, we must qualify this simple explanation of our results for the following reasons. Vinculin is present at cell-cell zonula adherens junctions in the absence of talin (Geiger et al., 1985). Earlier experiments on the reincorporation of vinculin into extracted focal contacts indicated that vinculin could bind to other proteins, in addition to talin (Ball et al., 1986). Also, deletion of only the talin-binding sequence in the above transfection experiments did not prevent the incorporation of the expressed vinculin into focal contacts. The latter result was interpreted (Bendori et al., 1989) in terms of the ability of vinculin to self-associated tail-to-tail (Molony and Burridge, 1985). However, self-association can account for only the continued, not initial, accumulation of vinculin at the focal contact. In this context, the isolation of a new vinculin-binding focal contact protein, paxillin, is of interest (Turner et al., 1990). Paxillin binds to the vinculin tail-piece. It apparently does not bind to talin, filamin, α -actinin, or actin in blot overlay assays, but binding to membrane proteins, such as integrins, was not probed in these assays (Turner et al., 1990). Thus, paxillin could bind to the focal contact membrane and provide a route paralleling that of talin for the initial incorporation of vinculin at the focal contact. It will be important to follow the time course of accumulation of

paxillin relative to vinculin and talin at the focal contact to determine whether such a parallel route is possible.

A final question raised by this study concerns the accumulation of vinculin with talin at new focal contacts, but not with the talin only a few micrometers away in the nodes at the edge of the lamellipodium. It is unlikely that the molecular size and configuration of vinculin, with an 8-nm globular head and 20-nm tail-piece (Molony and Burridge, 1985), result in the physical exclusion of vinculin from the lamellipodium, since ficoll molecules with a 3.5-nm, but not 25-nm, hydrodynamic radius penetrate these structures (Luby-Phelps and Taylor, 1988). The accumulation of vinculin only at the focal contact suggests a higher affinity of the binding site on talin for vinculin or an increased accessibility of the site at the focal contact. The basis for such an adhesion-dependent regulation of talin activity is unclear. However, it must be considered a consequence of the sequence of regulatory steps that control the initial accumulation of talin at the focal contact versus those that control the adhesion-independent accumulation of talin in nodes at the edge of the lamellipodium.

We are indebted to Drs. Keith Burridge and Irwin Singer for their generous gifts of the rabbit antitalin serum and guinea pig antivinculin serum, respectively.

This investigation was supported by National Science Foundation grant, DCB-8702511, to C. S. Izzard.

Received for publication 18 January 1991 and in revised form 12 March 1991.

References

- Abercrombie, M., and G. A. Dunn. 1975. Adhesions of fibroblasts to substratum during contact inhibition observed by interference reflection microscopy. *Exp. Cell Res.* 92:57-62.
- Abercrombie, M., J. E. M. Heaysman, and S. M. Pegrum. 1971. The locomotion of fibroblasts in culture. IV. Electron microscopy of the leading edge. *Exp. Cell Res.* 67:359-367.
- Ball, E. H., C. Freitag, and S. Gurofsky. 1986. Vinculin interaction with permeabilized cells: disruption and reconstitution of a binding site. *J. Cell Biol.* 103:641-648.
- Beckerle, M. C. 1990. The adhesion plaque protein, talin, is phosphorylated in vivo in chicken embryo fibroblasts exposed to a tumor-promoting phorbol ester. *Cell Regulation.* 1:227-236.
- Bendori, R., D. Salomon, and B. Geiger. 1989. Identification of two distinct functional domains on vinculin involved in its association with focal contacts. *J. Cell Biol.* 108:2383-2393.
- Bretscher, A., and K. Weber. 1980. Fimbrin, a new microfilament-associated protein present in microvilli and other cell surface structures. *J. Cell Biol.* 86:335-340.
- Buck, C. A., and A. F. Horwitz. 1987. Integrin, a transmembrane complex mediating cell-substratum adhesion. *J. Cell Sci. Suppl.* 8:231-250.
- Burn, P., A. Kupfer, and S. J. Singer. 1988. Dynamic membrane-cytoskeletal interactions: specific association of integrin and talin arises in vivo after phorbol ester treatment of peripheral blood lymphocytes. *Proc. Natl. Acad. Sci. USA.* 85:497-501.
- Burridge, K., and L. Connell. 1983a. Talin: a cytoskeletal component concentrated in adhesion plaques and other sites of actin-membrane interaction. *Cell Motil. Cytoskeleton.* 3:405-417.
- Burridge, K., and L. Connell. 1983b. A new protein of adhesion plaques and ruffling membranes. *J. Cell Biol.* 97:359-367.
- Burridge, K., and P. Mangeat. 1984. An interaction between vinculin and talin. *Nature (Lond.).* 308:744-746.
- Burridge, K., and L. Molony. 1990. Talin: biochemistry and cell biology. *Adv. Cell Biol.* 3:95-109.
- Burridge, K., K. Fath, T. Kelly, G. Nuckolls, and C. Turner. 1988. Focal adhesions: transmembrane junctions between the extracellular matrix and the

- cytoskeleton. *Annu. Rev. Cell Biol.* 4:487-525.
- Chen, W.-T., E. Hasegawa, T. Hasegawa, C. Weinstock, and K. M. Yamada. 1985. Development of cell surface linkage complexes in cultured fibroblasts. *J. Cell Biol.* 100:1103-1114.
- DePasquale, J. A., and C. S. Izzard. 1987. Evidence for an actin-containing precursor of the focal contact and the timing of incorporation of vinculin at the focal contact. *J. Cell Biol.* 105:2803-2809.
- Drenckhahn, D., M. Beckerle, K. Burridge, and J. Otto. 1988. Identification and subcellular location of talin in various cell types and tissues by means of [¹²⁵I] vinculin overlay, immunoblotting and immunocytochemistry. *Eur. J. Cell Biol.* 46:513-522.
- Fath, K. R., C.-R. S. Edgell, and K. Burridge. 1989. The distribution of distinct integrins in focal contacts is determined by the substratum composition. *J. Cell Sci.* 92:67-75.
- Geiger, B. 1979. A 130K protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. *Cell.* 18:193-205.
- Geiger, B., T. Volk, and T. Volberg. 1985. Molecular heterogeneity at adhesion junctions. *J. Cell Biol.* 101:1523-1531.
- Heath, J. P., and G. A. Dunn. 1978. Cell to substratum contacts of chick fibroblasts and their relation to the microfilament system. A correlated interference-reflexion and high-voltage electron-microscope study. *J. Cell Sci.* 29:197-212.
- Höglund, A.-S., R. Karlsson, E. Arro, B.-A. Fredriksson, and U. Lindberg. 1980. Visualization of the peripheral weave of microfilaments in glial cells. *J. Musc. Res. Cell Motil.* 1:127-146.
- Horwitz, A., K. Duggan, C. Buck, M. C. Beckerle, and K. Burridge. 1986. Interaction of plasma membrane fibronectin receptor with talin—a transmembrane linkage. *Nature (Lond.).* 320:531-533.
- Izzard, C. S. 1988. A precursor of the focal contact in cultured fibroblasts. *Cell Motil. Cytoskeleton.* 10:137-142.
- Izzard, C. S., and L. R. Lochner. 1976. Cell-to-substrate contacts in living fibroblasts: an interference reflexion study with an evaluation of the technique. *J. Cell Sci.* 21:129-159.
- Izzard, C. S., and L. R. Lochner. 1980. Formation of cell-to-substrate contacts during fibroblast motility: an interference reflexion study. *J. Cell Sci.* 42:81-116.
- Jones, P., P. Jackson, G. J. Price, B. Patel, V. Ohanian, A. L. Lear, and D. R. Critchley. 1989. Identification of a talin binding site in the cytoskeletal protein vinculin. *J. Cell Biol.* 109:2917-2927.
- Lazarides, E. 1976. Two general classes of cytoplasmic actin filaments in tissue culture cells: the role of tropomyosin. *J. Supramol. Struct.* 5:531-563.
- Luby-Phelps, K., and D. L. Taylor. 1988. Subcellular compartmentalization by local differentiation of cytoplasmic structure. *Cell Motil. Cytoskeleton.* 10:28-37.
- Molony, L., and K. Burridge. 1985. Molecular shape and self-association of vinculin and metavinculin. *J. Cell. Physiol.* 29:31-36.
- Molony, L., D. McCaslin, J. Abernethy, B. Paschal, and K. Burridge. 1987. Properties of talin from chicken gizzard smooth muscle. *J. Biol. Chem.* 262:7790-7795.
- Mueller, S. C., T. Hasegawa, S. S. Yamada, K. M. Yamada, and W.-T. Chen. 1988. Transmembrane orientation of the fibronectin receptor complex (integrin) demonstrated directly by a combination of immunocytochemical approaches. *J. Histochem. Cytochem.* 36:297-306.
- Mueller, S. C., T. Kelly, M. Dai, H. Dai, and W.-T. Chen. 1989. Dynamic cytoskeleton-integrin associations induced by cell binding to immobilized fibronectin. *J. Cell Biol.* 109:3455-3464.
- Muguruma, M., S. Matsumura, and T. Fukuzawa. 1990. Direct interaction between talin and actin. *Biochem. Biophys. Res. Commun.* 171:1217-1223.
- Otey, C. A., F. M. Pavalko, and K. Burridge. 1990. An interaction between α -actinin and the β_1 integrin subunit in vitro. *J. Cell Biol.* 111:721-729.
- Otto, J. J. 1990. Vinculin. *Cell Motil. Cytoskeleton.* 16:1-6.
- Small, J. V. 1981. Organization of actin in the leading edge of cultured cells: influence of osmium tetroxide and dehydration on the ultrastructure of actin meshworks. *J. Cell Biol.* 91:695-705.
- Small, J. V., G. Rinnerthaler, and H. Hinssen. 1982. Organization of actin meshworks in cultured cells: the leading edge. *Cold Spring Harbor Symp. Quant. Biol.* 46:599-611.
- Tidball, J. G., T. O'Halloran, and K. Burridge. 1986. Talin at myotendinous junctions. *J. Cell Biol.* 103:1465-1472.
- Turner, C. E., J. R. Glenney, Jr., and K. Burridge. 1990. Paxillin: a new vinculin-binding protein present in focal adhesions. *J. Cell Biol.* 111:1059-1068.
- Wehland, J., M. Osborn, and K. Weber. 1979. Cell-to-substratum contacts in living cells: a direct correlation between interference-reflexion and indirect-immunofluorescence microscopy using antibodies against actin and α -actinin. *J. Cell Sci.* 37:259-273.