Dynamic Cytoskeleton–Integrin Associations Induced by Cell Binding to Immobilized Fibronectin

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Abstract. We have examined the early events of cellular attachment and spreading (10–30 min) by allowing chick embryonic fibroblasts transformed by Rous sarcoma virus to interact with fibronectin immobilized on matrix beads. The binding activity of cells to fibronectin beads was sensitive to both the mAb JG22E and the GRGDS peptide, which inhibit the interaction between integrin and fibronectin. The precise distribution of cytoskeleton components and integrin was determined by immunocytochemistry of frozen thin sections. In suspended cells, the distribution of talin was diffuse in the cytoplasm and integrin was localized at the cell surface. Within 10 min after binding of cells and fibronectin beads at 22°C or 37°C, integrin and talin aggregated at the membrane adjacent to the site of bead attachment. In addition, an internal pool of integrin-positive vesicles accumulated. The mAb ES238 directed against the extracellular domain of the avian β_1 integrin subunit, when coupled to beads, also induced the aggregation of talin at the membrane, whereas ES186 directed against the intracellular domain of the β_1 integrin subunit did not. Cells attached and spread on Con A beads, but neither integrin nor talin aggregated at the membrane. After 30 min, when many of the cells were at a more advanced stage of spreading around beads or phagocytosing beads, α-actinin and actin, but not vinculin, form distinctive aggregates at sites along membranes associated with either fibronectin or Con A beads. Normal cells also rapidly formed aggregates of integrin and talin after binding to immobilized fibronectin in a manner that was similar to the transformed cells, suggesting that the aggregation process is not dependent upon activity of the pp60$^{v\text{-}src}$ tyrosine kinase. Thus, the binding of cells to immobilized fibronectin causes integrin–talin coaggregation at the sites of membrane–ECM contact, which can initiate the cytoskeletal events necessary for cell adhesion and spreading.

The cell receptor for fibronectin, a member of the integrin family (Hynes, 1987; Buck and Horwitz, 1987; Ruoslahti and Pierschbacher, 1987; Yamada, 1988), interacts with the extracellular matrix (ECM)[1] and the cytoskeleton, and is thus thought to play a critical role in the transmembrane control of adhesion of cells to their environment as well as in cell motility (for reviews see DeSimone and Hynes, 1988; Yamada, 1988). Integrin present in avian fibroblasts consists of three or more glycoproteins, at least three α and one β subunit linked noncovalently in heterodimers (Hasegawa et al., 1985; Hynes, 1987; Hynes et al., 1989; Knudsen et al., 1985). Only the oligomer is active in binding its ligand (Buck et al., 1986). The avian β subunit is a member of the β_1 subfamily of integrins that includes mammalian fibronectin (FN) receptors, the immune system very late antigens (VLA 1-6), and platelets GPIa/IIa and GPIc/IIa (Akiyama et al., 1989; Hemler, 1988; Hynes, 1987; Kunicki et al., 1988; Pischel et al., 1988; Ruoslahti and Pierschbacher, 1987; Yamada, 1988). The β subunit (band 3) is transmembrane (Mueller et al., 1988; Tamkun et al., 1986), as are all of the α subunits sequenced to date so that the integrin complex is in a position to mediate interactions between the ECM and cytoskeleton.

The mechanisms by which integrin influences transmembrane interactions between the cytoskeleton and the ECM are not fully understood. Avian integrin has been observed in vitro to bind the extracellular proteins FN (Hasegawa et al., 1985; Horwitz et al., 1986*a*), laminin and vitronectin (Buck and Horwitz, 1987; Horwitz et al., 1986*a*), as well as the cytoskeletal protein talin (Horwitz et al., 1986*b*). In transformed cells, integrin is available for interaction with exogenously added cellular FN to form transmembrane FN-integrin-actin colocalizing complexes (Chen et al., 1986*b*; Roman et al., 1989), suggesting that ECM proteins initiate transmembrane events including association of cytoskeleton with the membrane (Rinnerthaler et al., 1988: Geiger et al., 1984). The synthetic peptide Gly-Arg-Gly-Asp-Ser (GRGDS), derived from the sequence of the cell-binding region of FN, can prevent the formation of these cell surface linkage com-

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1. *Abbreviations used in this paper*: CEF, chicken embryo fibroblast; ECM, extracellular matrix; FN, fibronectin; RSV, Rous sarcoma virus; RSVCEFs, Rous sarcoma virus–transformed chicken embryo fibroblasts.

plexes (Chen et al., 1986*b*). Disrupting integrin's association with the substratum with GRGDS peptide causes the dissociation of cytoskeletal α-actinin and vinculin from contact sites (Stickel and Wang, 1988). Conversely, events within the cytoplasm may be necessary for differentiation of adhesive sites upon contact with the substratum (DePasquale and Izzard, 1987; Izzard and Lochner, 1980). Other biochemical events within the cytoplasm may be prerequisite for the establishment of cytoskeleton-membrane linkages and for the aggregation of FN and its receptor on the cell surface. Recently, Burn et al. (1988) found that antibodies directed against integrin cause the coaggregation of talin and integrin into caps only after stimulation of lymphocytes with PMA, which might alter the phosphorylation state of integrin. Integrin subunits are phosphorylated on tyrosine after Rous sarcoma virus (RSV) transformation, and the binding activity of the phosphorylated form for FN and talin has been suggested to be greatly reduced (Buck and Horwitz, 1987; Hirst et al., 1986; Tapley et al., 1989). Integrin-containing transmembrane linkages may thus be subject to regulation by phosphorylation so that cellular adhesions can be made and broken during the motility of a cell. In addition, integrin is involved in tissue stability by forming firm cell–matrix adhesions in differentiating cells. This is particularly evident at the cell–ECM contact sites of cultured stationary cells where there are prominent associations with both FN and the cytoskeleton (Chen et al., 1985*b*; Damsky et al., 1985). Integrin has been shown to be relatively immobile in these contact sites (Duband et al., 1988).

To examine possible transmembrane signaling induced by the ECM, we have developed cross-linked gelatin matrix beads to which FN or other matrix proteins can be covalently immobilized for studies of localized cellular responses. Chicken embryonic fibroblasts (CEFs) transformed by RSV (RSVCEFs) were chosen initially because these cells synthesize FN at low levels and show a nearly homogeneous pattern of integrin distribution over the membrane surface (Chen et al., 1986*b*). Previously, latex beads coated with various proteins have been used to study the properties of binding, phagocytosis, and recycling of cell surface receptors mediated by FN, vitronectin, monoclonal antibodies against cell surface components, and Con A (Grinnell and Geiger, 1986; McAbee and Grinnell, 1983, 1985; Grinnell et al., 1988; Molnar et al., 1987; Wagner, 1982). In addition to avoiding possible charge effects presented by other bead surfaces, such as derivatized latex beads, the gelatin beads used in this study allow a covalent linkage of proteins to the bead surface. And, they permit a facile observation of cellular responses to FN–bead binding by frozen thin sectioning and immunofluorescence techniques at very early times after cell contact. Using these methods, we have demonstrated that cellular binding of exogenous cross-linked FN induces rapid recruitment of integrin–talin complexes and their associated cytoskeleton to the membrane at the site of cell–bead contact in both normal and transformed CEFs. Moreover, this rapid recruitment of talin is specific for integrin binding to FN because we show it also can be induced with immobilized antibodies directed against integrin, and bead binding can be inhibited by JG22E and the GRGDS peptide. The aggregation of integrin and talin at contact sites probably plays a significant early role in modulation of cell surface movement during attachment and spreading.

Materials and Methods

Cell Culture

CEFs and RSVCEFs were cultured as described (Chen et al., 1984; Olden and Yamada, 1977). Cells were suspended using trypsin (Gibco Laboratories, Grand Island, NY), and resuspended in serum-free medium (DME/F12, 1:1 [Gibco Laboratories], 20 mM Hepes, pH 7.1, ITS Premix [used as directed; Collaborative Research, Inc., Bedford, MA], 0.04 mg/ml BSA, 100 U/ml streptomycin, 100 μg/ml penicillin, 2 mM glutamine).

Cross-linked Gelatin Beads

Cross-linked gelatin beads were prepared as follows: 0.15 ml of a solution of 5% gelatin, 5% sucrose, in PBS (0.02 M phosphate buffer, pH 7.4, 0.15 M NaCl) at 50°C was added to 0.5 ml water-saturated 1-butanol (Fisher Scientific Co., Pittsburgh, PA) in a 1.5-ml microfuge tube, vortexed for 10 s, and sonicated for 10 s at a setting of No. 30 on a microprobe-type ultrasonic micro cell disrupter (Kontes Co., Vineland, NJ). The mixture was left undisturbed on ice for 15 min; after the addition of 0.8 ml 5% glutaraldehyde in PBS at 4°C, the mixture was vortexed and left undisturbed on ice for 1 h. After centrifugation for 1 min in a microcentrifuge (model 59A, Fisher Scientific Co.) setting No. 10, the supernate was removed and the bead pellet was sonicated in 1 ml PBS and washed once. Large beads (more than 20 μm in diameter) were removed by a 5-s centrifugation at setting No. 2. The supernate was then centrifuged for 30 s at setting No. 2 to obtain beads with an average diameter of 10 μm. Approximately 10^7 beads were incubated with 1 ml 50 μg/ml human plasma FN (New York Blood Bank, New York, NY), 100 μl 1 mg/ml laminin (Bethesda Research Laboratories, Gaithersburg, MD), 50 μl 1 mg/ml Con A (Sigma Chemical Co., St. Louis, MO), or 1 ml 50 μg/ml BSA (Sigma Chemical Co.) for 1 h at 22°C using end-over-end mixing. Alternatively, beads were incubated overnight at 4°C with 200 μl 0.25 mg/ml ES238 or ES186 rat IgG (Mueller et al., 1988). Beads that had been protein cross-linked were recovered by centrifugation and resuspended by sonication in serum-free medium, and they were either used immediately or stored at −20°C in a 1:1 mixture of glycerol and serum-free medium.

Cell–Bead Incubation and Fixation

CEFs or RSVCEFs and beads at 3.5–10 × 10^5 cells/ml and 1–2 × 10^6 beads/ml were incubated using end-over-end mixing at 22°C for 20 min or for the times and temperatures indicated, collected by centrifugation, washed, and fixed by the addition of 9% paraformaldehyde in PBS, pH 7.4, to a final concentration of 3% (Chen et al., 1985*a*).

RSVCEFs (7.0 × 10^5 cells/ml) were preincubated for 45 min at 37°C with inhibitory agents (GRGDS or GRGES [0.15 mg/ml]), for an additional 20 min at 22°C with beads (1.3 × 10^6 beads/ml), and then fixed; and the percent of cells with beads was determined using phase-contrast microscopy (25× Plan-Neofluar) to detect cells and fluorescence microscopy to detect the autofluoresence of beads (see Fig. 2). Next, RSVCEFs (3.5 × 10^5 cells/ml) were preincubated for 30 min at 37°C with inhibitory agents (JG22E [0.1 mg/ml], ES238 [0.1 mg/ml], or ES186 [0.1 mg/ml]), for an additional 20 min at 22°C with beads (2 × 10^6 beads/ml), and then fixed; and the concentration of unbound cells was determined using a hemocytometer and phase-contrast microscopy (see Fig. 3). We found that the method of quantification most appropriate for measuring bead–cell interaction was dependent upon the degree of cellular aggregation caused by the beads. In the case that aggregates contained no more than 2–3 cells, the method of counting cells with bound beads using fluorescence microscopy was most appropriate (see Fig. 2). In the case that aggregates contained more than three cells, it was more accurate to measure the concentration of cells remaining unbound (see Fig. 3). Measurements were done in triplicate and the *t* test was used to determine whether differences between means were significant.

Mouse mAb JG22E (Greve and Gottlieb, 1982; Chen et al., 1985*a*) was purified from ascites by protein A chromatography using the Pierce Monopure system (Pierce Chemical Co., Rockford, IL), and ES238 was prepared as previously described (Mueller et al., 1988). GRGES and GRGDS were purified by HPLC and were a kind gift from Dr. Kenneth M. Yamada (Laboratory of Molecular Biology, National Cancer Institute, Bethesda, MD).

Frozen Thin Sectioning and Immunofluorescence Microscopy

Fixed cells bound to beads that had been protein cross-linked were embed-

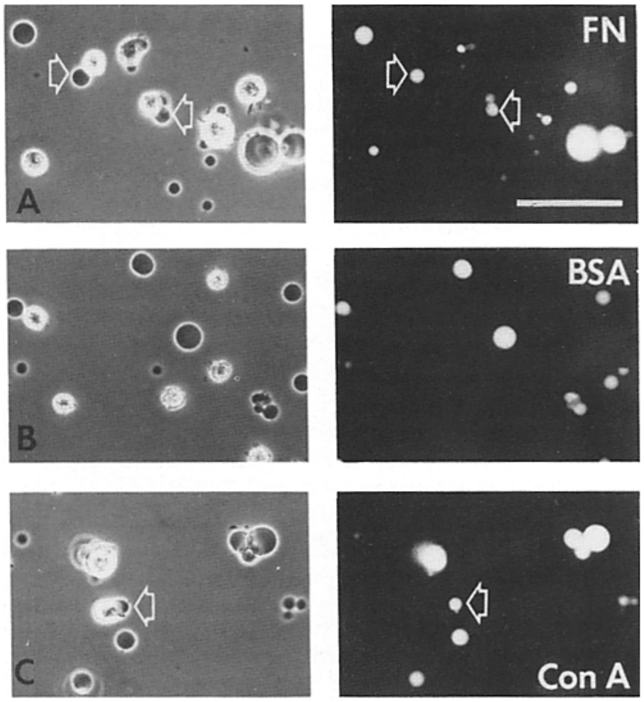

Figure 1. Binding of RSVCEFs with FN beads (*A*), BSA beads (*B*), and Con A beads (*C*). Cells were incubated with beads in suspension at 37°C for 10 min. At left, phase-contrast micrographs show examples of beads bound to cells (*arrows*). At right, fluorescence micrographs of the same field show fluorescent images of glutaraldehyde-fixed beads. The same cell-bead aggregates are indicated by arrows. FN beads (*A*) and Con A beads (*C*) rapidly aggregated with cells while BSA beads (*B*) did not. Similar results are obtained at 22°C. Bar, 50 μm.

ded in 5% gelatin and processed for 0.5-μm frozen thin sectioning and immunofluorescence microscopy as previously described (Chen et al., 1985*a*, 1986*a*). Integrin β_1 was detected using rat mAb ES238, directed against an extracellular epitope of β_1 (Mueller et al., 1988). Talin was detected using affinity-purified anti-190K (Mueller et al., 1988), a gift of Dr. Shinsuke Saga (Nagoya University, School of Medicine, Nagoya, Japan), or rabbit antitalin antisera, a kind gift of Dr. Keith Burridge (University of North Carolina, Chapel Hill, NC). Alpha-actinin was detected using affinity-purified rabbit anti-α-actinin (Chen and Singer, 1982), actin by rabbit anti-actin (ICN Biochemicals Inc., Irvine, CA), and vinculin using affinity-purified rabbit antivinculin (Chen and Singer, 1982). Frozen thin sections of cell-bead bindings were observed with phase contrast and immunofluorescence microscopy (Chen et al., 1985*a*) using a photomicroscope (model III; Carl Zeiss, Inc., Thornwood, NY).

Results

Specificity of Cell Binding to FN Beads

In this study, cells were incubated in suspension using serum-free medium for short (10–45 min) durations so that initial responses to bead–cell attachment might be examined. Beads cross-linked with either FN or Con A aggregated with cells, but those cross-linked with BSA did not (Fig. 1). Binding of cells and FN beads appeared to be temperature dependent. Using equal concentrations of beads and cells ($\sim$1 × 10^6/ml), the percentage of bound beads was measured. After 30 min, < 5% of FN beads were bound at 4°C, whereas 28% were bound after 20 min at 22°C, and 72% were bound after 10 min at 37°C. At these times, the majority of the beads were still extracellular, whereas at later times, smaller beads were phagocytosed. Only 6% of BSA beads were bound after 10 min at 37°C.

To investigate the role of integrin in binding, we looked at binding inhibition by the FN cell-binding peptide GRGDS, and by the adhesion inhibiting, antireceptor mAb JG22E (Chen et al., 1985*a*). GRGDS significantly ($p < 0.0025$) reduced the number of cells with FN beads relative to GRGES. There was no significant effect of GRGDS on the number of cells with bound laminin beads, Con A beads, or BSA beads (Fig. 2). In this experiment, laminin-bead binding was not significantly higher than BSA-bead binding. JG22E inhibits cell binding to FN beads relative to either ES186, an mAb directed against an intracellular epitope of integrin β_1 ($P < 0.001$), or ES238, a noninhibitory mAb directed against an extracellular epitope of integrin β_1 ($P < 0.001$) (Mueller et al., 1988). JG22E, ES238, and ES186 monoclonals had no significant effect on cell binding to Con A beads or to laminin beads (Fig. 3). Taken together, it appears likely that FN–bead binding to cells within the 20-min incubation is largely mediated by integrin, since both JG22E and GRGDS inhibited FN-bead binding.

Contact of Transformed Cells with FN Beads Induces the Aggregation of Integrin and Talin at Contact Sites

RSVCEFs were prepared for 0.5-μm frozen thin sectioning before or after binding of cells to beads, and examined by immunofluorescence microscopy (Fig. 4). The localization of integrin and talin was examined after incubating cells without beads for 0 or 30 min after trypsinization in serum-free medium at 22°C (Fig. 4 *C*). In the presence of calcium and magnesium, integrin is relatively insensitive to proteolytic digestion (Mueller et al., 1988); however, the trypsin treatment effectively removes extracellular FN from the cell surface as shown by immunofluorescence microscopy using fluorescein goat antifibronectin (not shown). Integrin was localized almost exclusively at the cell surface in cells suspended in serum-free medium for 0 (not shown) or 30 min after trypsinization (Fig. 4 *C*, *arrow*), whereas talin labeling was predominantly diffuse in the cytoplasm (Fig. 4 *C*).

Two noteworthy changes took place upon binding of FN

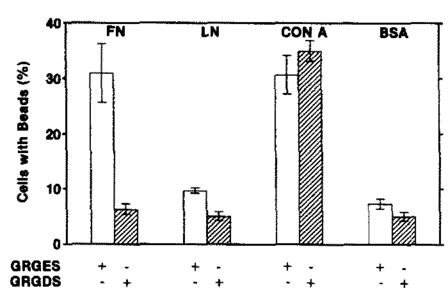

Figure 2. Inhibition of FN–bead binding to cells by GRGDS. Cells and beads were incubated as described in Materials and Methods beginning with a preincubation with 0.15 mg/ml GRGDS, the cell-binding peptide derived from FN, or GRGES, the control peptide, at 37°C followed by binding at 22°C. GRGDS significantly ($P < 0.0025$) reduced the number of cells with FN beads (*FN*), but had no significant effect on the number of cells with bound Con A beads (*CON A*). Laminin–bead binding (*LN*) was not significantly higher than BSA-bead binding (*BSA*).

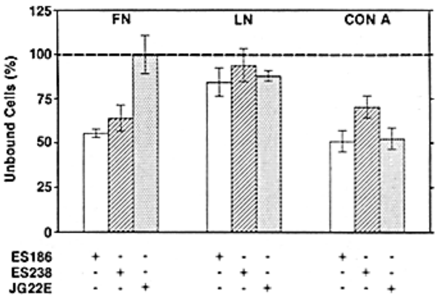

Figure 3. Inhibition of FN–bead binding by JG22E. Cells and beads were incubated as described in Materials and Methods. JG22E, an adhesion-inhibiting mAb directed against an extracellular epitope of integrin β_1, inhibits cell binding to FN beads (*FN*) relative to either ES186, a mAb directed against an intracellular epitope of integrin β_1 ($P < 0.001$), or ES238, a non-inhibitory mAb directed against an extracellular epitope of integrin β_1 ($P < 0.001$). JG22E, ES238, and ES186 monoclonals had no significant effect on cell binding to Con A beads (*CON A*) or to laminin beads (*LN*).

beads to RSVCEFs. First, integrin and talin formed aggregates at sites of cell attachment to FN beads (Fig. 4, *A* and *B*). Second, an internal pool of vesicular integrin labeling was apparent in most cells within 10 min after contacting FN beads (Fig. 4, *A* and *B*). Talin distribution was quite intense in the cytoplasm adjacent to the bead-bound plasma membrane but talin did not always colocalize with cytoplasmic vesicular integrin staining (Fig. 4, *A* and *B*). Similar integrin–talin coaggregation was observed after incubation of RSVCEFs and FN beads for 10 min at 37°C compared to incubation for 20 min at 22°C (not shown).

In contrast, integrin was distributed over the entire surface of cells spreading on Con A beads with no increase in concentration at Con A–bead attachment sites (Fig. 5, *A* and *B*). Talin did not aggregate at the membrane adjacent to sites of cell attachment to Con A beads (Fig. 5, *A* and *B*). In addition, the vesicular integrin pool that was observed in the cytoplasm of cells contacting FN beads was not apparent in cells contacting Con A beads (Fig. 5). Thin cellular extensions were often found surrounding Con A beads (Fig. 5 *B*, *arrowheads*) but were absent in Con A beads free of contact with cells. These cellular extensions that label positively for integrin, but negatively for talin, represent the cell surface where integrin and talin are uncoupled.

Distribution of Integrin, Talin, and the Cytoskeleton Proteins Vinculin, α-Actinin, and Actin in Transformed Cells Bound to FN Beads

In contrast to talin aggregation at the membrane (Fig. 4), vin-

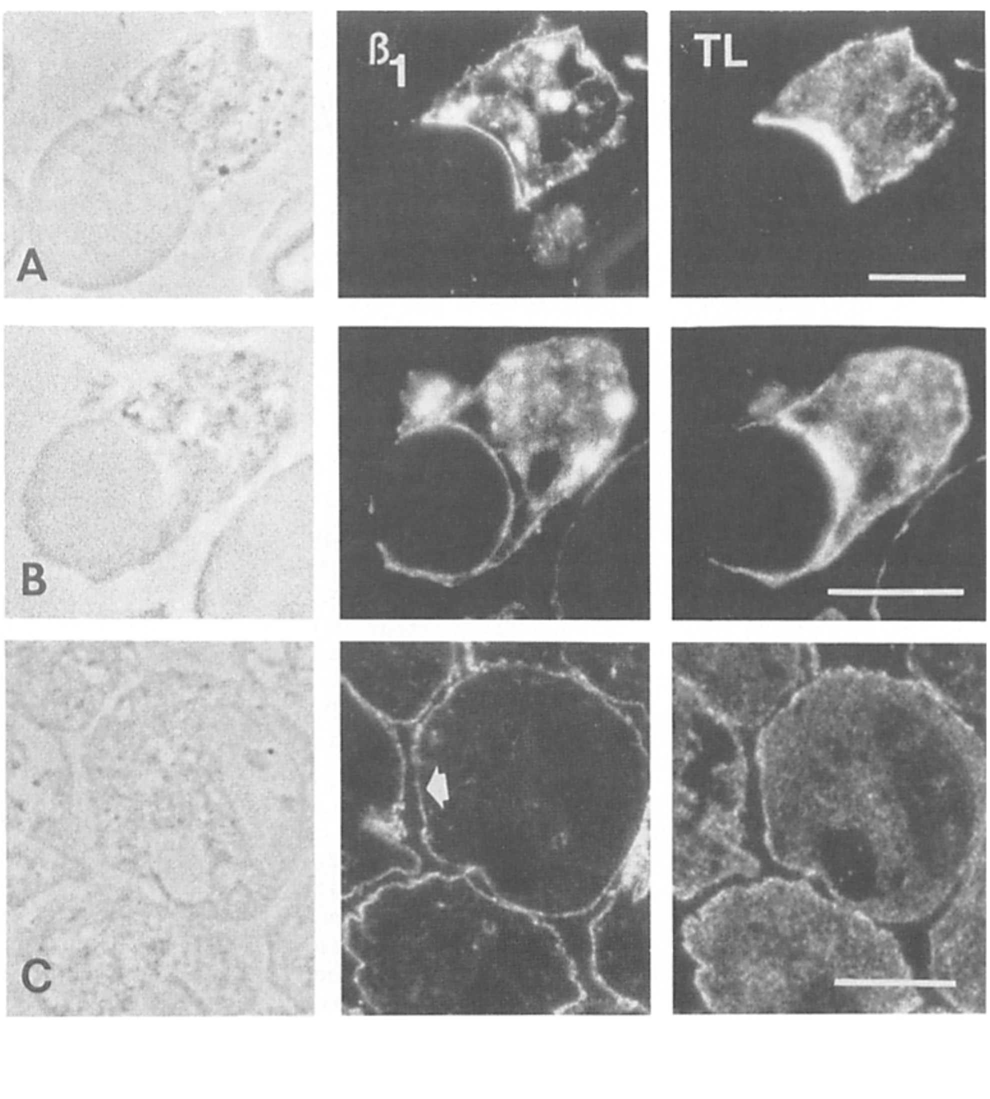

Figure 4. Aggregation of integrin (β_1) and talin (*TL*) at initial sites of cell attachment to the FN bead. Cells and FN beads were incubated for 20 min at 22°C and processed as described in Materials and Methods. The 0.5-μm frozen thin sections were double labeled for integrin β_1 (β_1) and talin (*TL*). Phase-contrast micrographs are shown at left (*A* and *B*) During several initial stages of spreading of RSVCEFs on FN beads, integrin (β_1), observed using mAb ES238, is aggregated at the membrane in contact with the bead and a prominent internal pool of integrin-positive vesicles is apparent. Talin is aggregated adjacent to the bead but frequently does not colocalize with integrin intracellularly. (*C*) The distribution of integrin (*center*) and talin (*right*) on cells in suspension in serum-free medium 30 min after trypsinization is shown. Integrin is localized at the cell surface (*center*; *arrows*), while talin labeling is diffuse in the cytoplasm (*right*). Bars, 10 μm.

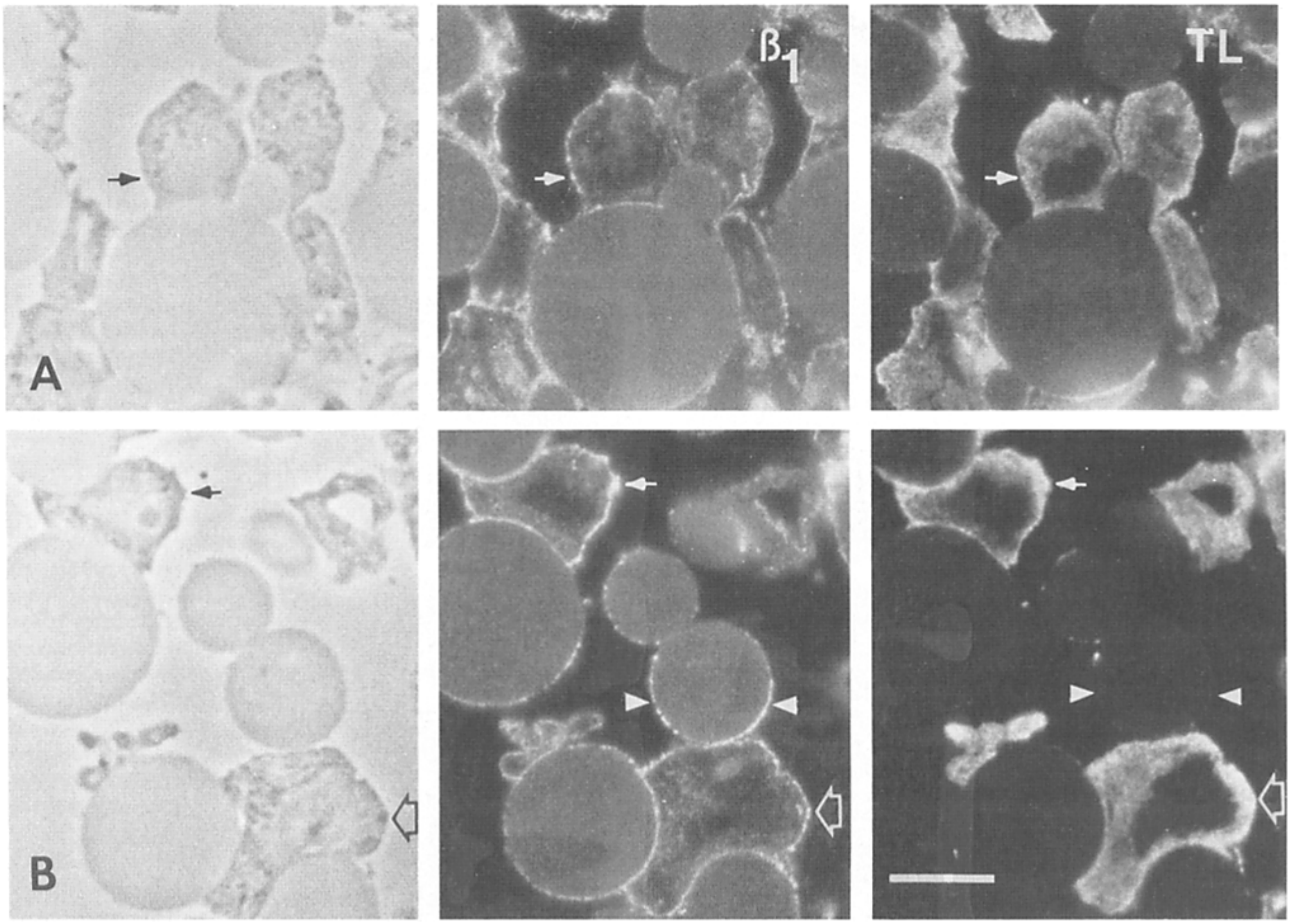

Figure 5. Integrin (β_1) and talin (*TL*) organization in cells attaching to Con A beads. (*A*) RSVCEF attaching to or spreading around Con A beads during a 10-min incubation at 22°C. Neither integrin (β_1) nor talin (*TL*) aggregate at the membrane adjacent to sites of cell attachment to Con A beads. (*B*) Later, after a 20-min incubation, integrin (*center*) and talin (*right*) are distributed prominently on the cell surface that is free of contact with Con A beads (*arrows*, *open arrows*) and are not aggregated on membranes in contact sites. Cellular extensions surrounding Con A beads are more extensive and thinner than those of cells attaching to FN beads, and they label positively for integrin, but negatively for talin (*arrowheads*). Con A beads without cells do not stain for integrin. Note that an internal pool of integrin-labeled vesicles is not apparent after incubation of cells with Con A beads. Bar, 10 μm.

culin displayed a diffuse cytoplasmic staining and was not aggregated at the membrane of cells in contact with FN beads after 20 min, even though integrin was clearly more concentrated at the membrane adjacent to the FN bead compared to elsewhere on the membrane (Fig. 6 *A*). Within the 10-min incubation of cells and beads, talin aggregation at the membrane in contact with the FN bead was observed in 19 of 23 cells containing beads (e.g., Fig. 6 *B*). Only 5 of 21 cells containing bound beads showed α-actinin aggregation at the bead surface (e.g., Fig. 6 *C*). Actin patches were found in only 3 of 22 cells (a negative example is shown in Fig. 6 *D*). Thus, talin aggregation occurs first in the series of events involving cytoskeleton association with the cell surface.

No aggregation of vinculin at the membrane in contact with the FN bead was seen even after 30 min of incubation (Fig. 7 *A*), whereas it was common at this time to see large patches of both α-actinin and actin adjacent to the bead surface (Fig. 7, *B–D*). The staining patterns observed for α-actinin and actin were similar to each other (Figs. 6, *C* and *D* and 7 *D*), but quite dissimilar from that of integrin and talin (Figs. 4 and 6 *B*). In addition, at 10 and 30 min aggregates of α-actinin and actin were observed in association with membranes attached to either FN beads (Figs. 6 and 7) or Con A beads (not shown).

Immobilized Anti-integrin Also Induces Talin Aggregation at the Membrane in Contact with Beads

mAbs directed against integrin were substituted on beads for FN to test whether, in the absence of FN, aggregation of the receptor by a specific antibody could stimulate talin recruitment to the bead-binding site of the cell membrane. Purified rat mAb IgG was cross-linked to gelatin beads and the beads were incubated with cells for 45 min at 22°C. mAb ES238 (directed against an extracellular epitope of integrin β_1) immobilized on beads, efficiently mediated bead binding, and induced talin aggregation (Fig. 8) similar to that induced by FN beads (Figs. 5 and 6). However, ES186 directed against a cytoplasmic epitope of integrin β_1 was ineffective in promoting bead binding (not shown). Bright labeling of ES238 beads by fluorescein conjugates of the secondary anti–rat antibody demonstrated the presence of cross-linked antibody throughout the gelatin beads, but obscured direct observation of integrin aggregation in the membrane adjacent to the beads (Fig. 8). To rule out the possibility that FN

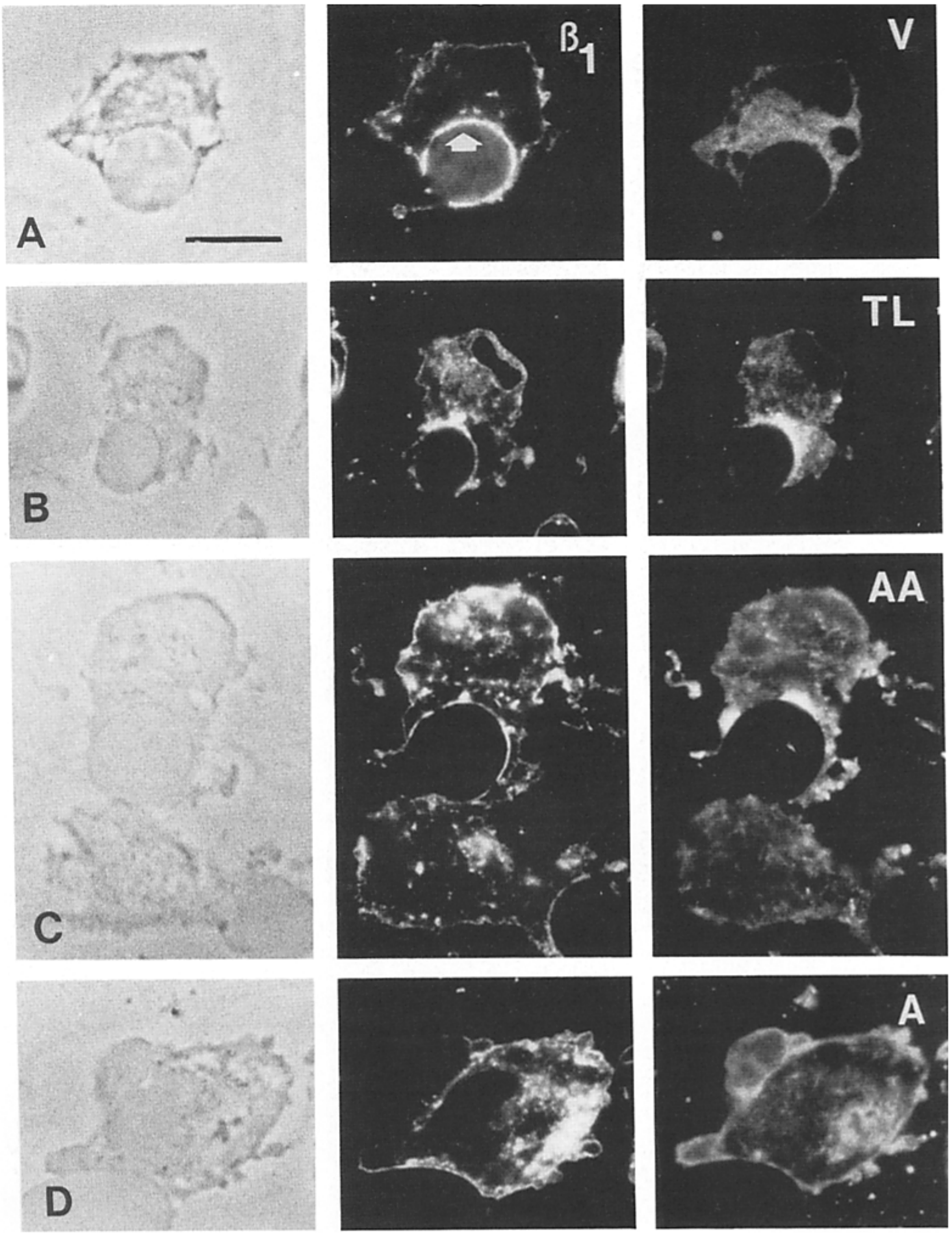

Figure 6. Aggregation of integrin (β_1), talin (*TL*), α-actinin (*AA*), and actin (*A*), but not vinculin (*V*) on membranes of transformed cells after incubation of FN beads and cells. (*A*) After a 20-min incubation of cells and FN beads at 22°C, vinculin (*V*) is not aggregated at the membrane adjacent to the bead even though the aggregation of integrin is quite apparent (*arrow*). (*B–D*) FN beads were incubated with cells for 10 min at 22°C and sections from the same block of cells were double labeled for integrin and cytoplasmic proteins. (*B*) Talin (*TL*) aggregation was found in 19 of 23 cells. In *C*, a cell containing the most intense α-actinin (*AA*) staining (the upper half) is shown as well as a cell containing no α-actinin patches (the lower half). In this experiment, 4 other cells of 21 total observed had α-actinin patches. (*D*) Actin patches were found in 3 of 22 cells, and an example of a cell with no actin aggregation is shown in the right panel. Intracellular vesicle staining for integrin is already apparent in cells incubated with FN beads for 10 min. Note in *B* and *C* that intracellular vesicles staining for integrin appear to be bound to the plasma membrane (*center panels*). Bar, 10 μm.

mediated the talin response of transformed cells attached to ES238 beads (i.e., was secreted, bound to the gelatin bead, and then bound integrin), transformed cells were incubated with ES238 beads for 20 min, and then sections were double labeled for talin and FN (Fig. 9). Fig. 9 shows that no FN labeling was observed on the surface of ES238 beads, although low levels of FN (relative to normal cells) were found intracellularly.

Integrin and Talin Coaggregate at the Membrane of Normal Cells in Contact with FN Beads

To investigate whether the formation of integrin–talin aggregates at FN-bead attachment sites is a transformation-specific phenomenon, we duplicated the experiments shown in Figs. 4 and 5 using normal, untransformed CEFs for 20 min at 22°C. We found that normal CEFs incubated with FN beads also demonstrated integrin–talin coaggregation at the bead–cell contact sites and an internal pool of receptor-positive material was apparent (Fig. 10, *A* and *B*). CEFs in suspension had integrin location at the cell surface and a diffuse intracellular talin distribution (Fig. 10 *C*) similar to that observed for RSVCEFs (Fig. 4 *C*). Integrin–talin coaggregation was not observed in CEFs attached to Con A beads for 20 min (not shown). In these experiments, 351 of 430 RSVCEFs (82%) and 144 of 278 CEFs (52%) in contact with FN beads had talin aggregates at the sites of membrane–bead contact.

Discussion

We have developed a gelatin bead method for immobilizing

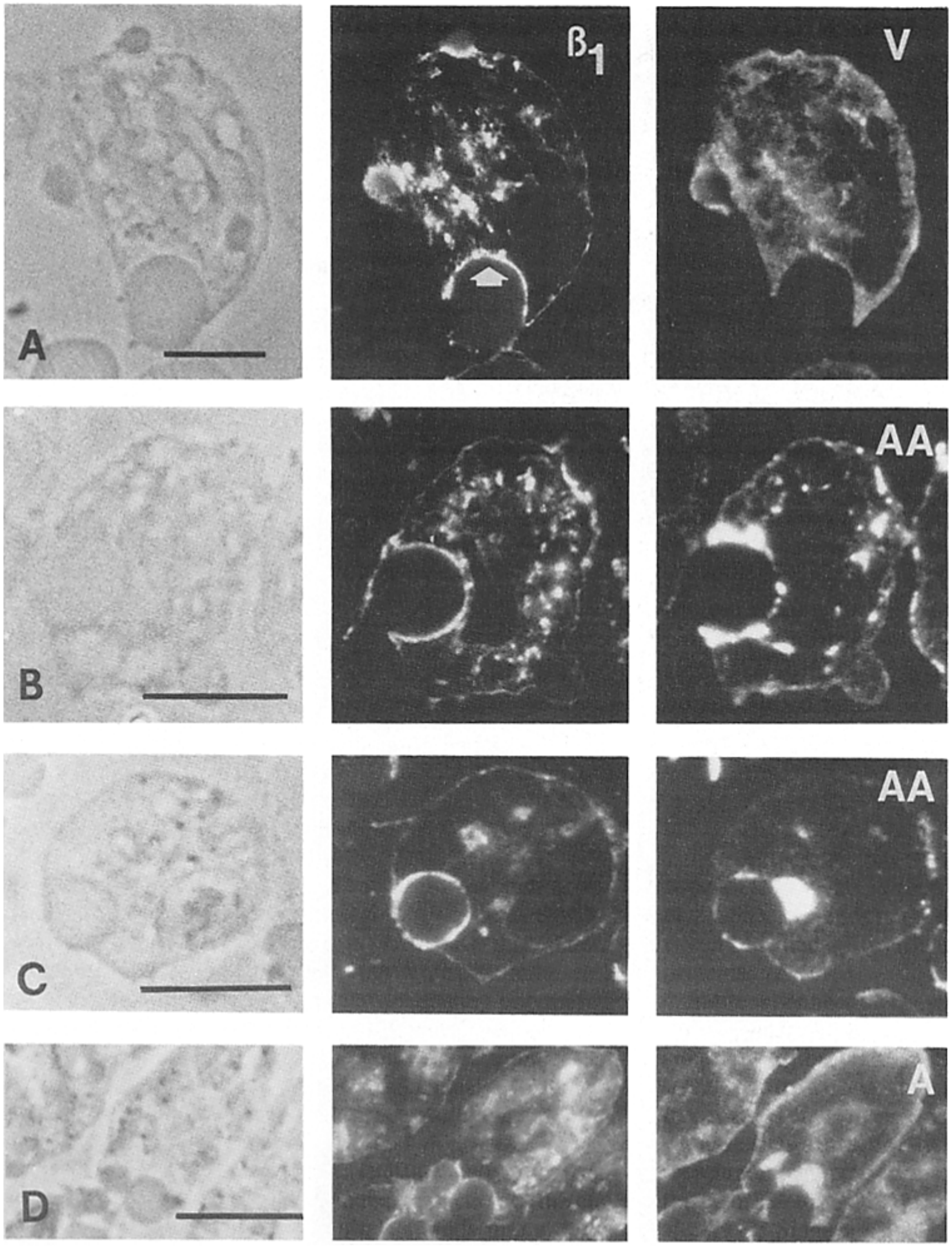

Figure 7. Aggregation of integrin (β_1), α-actinin (*AA*), and actin (*A*), but not vinculin (*V*), on membranes of transformed cells after incubation of FN-beads and cells for 30 min at 22°C. (*A*) Vinculin (*V*) is not aggregated at the membrane adjacent to the bead even though the aggregation of integrin is quite apparent (*arrow*). (*B* and *C*) Patches of α-actinin (*AA*) are found next to the FN bead (*right*), while integrin staining is intense around the bead (*center*), although slightly less so adjacent to the α-actinin patch in *C*. (*D*) Actin staining (*A*) shows similar characteristics to that described for α-actinin including the large patches at the bases of beads (*right*). Abundant intracellular vesicle staining for integrin is apparent when cells are incubated with FN beads (*A* and *D*). Bars, 10 μm.

FN (or other proteins) to examine the dynamic cellular responses elicited by contact of cells with FN substrata. Binding of the cells to FN beads, but not to Con A beads, induces the aggregation of integrin and talin at the contact sites and the appearance of an internal pool of integrin-labeled vesicles within 10 min at 22 and 37°C. That integrin mediates the response is supported by the fact that FN–bead binding is sensitive to mAb JG22E and GRGDS peptide, both of which inhibit the interaction between integrin and FN. And, directly aggregating integrin with the mAb ES238 also induces talin aggregation at membrane–bead contact sites. The ineffective binding of CEFs and RSVCEFs to the laminin beads is somewhat surprising, since integrin is reported to have laminin-binding properties (Gehlsen et al., 1988; Horwitz et al., 1986*a*; Ignatius and Reichardt, 1988).

Con A beads probably induce the aggregation of many glycoproteins in the membrane. In cells attached to Con A beads, however, integrin is not aggregated at bead contact sites, but is distributed over the entire cell membrane including the fine membrane protrusions surrounding beads where talin is distinctly absent (Fig. 5 *B, arrowheads*). Thus, integrin–talin associations are stimulated by cellular binding to FN, but not Con A.

We hypothesize that exogenous, cross-linked FN aggregates integrin which in turn stimulates the accumulation of membrane-associated talin and the formation of an intracellular pool of integrin-containing vesicles. Transformed cells do not synthesize enough or the right kind of FN to form an extracellular FN network. However, FN derived from normal cells can induce normal linkage complex formation in transformed cells (Chen et al., 1986*b*; Roman et al., 1989). The immobilization of plasma FN on gelatin beads

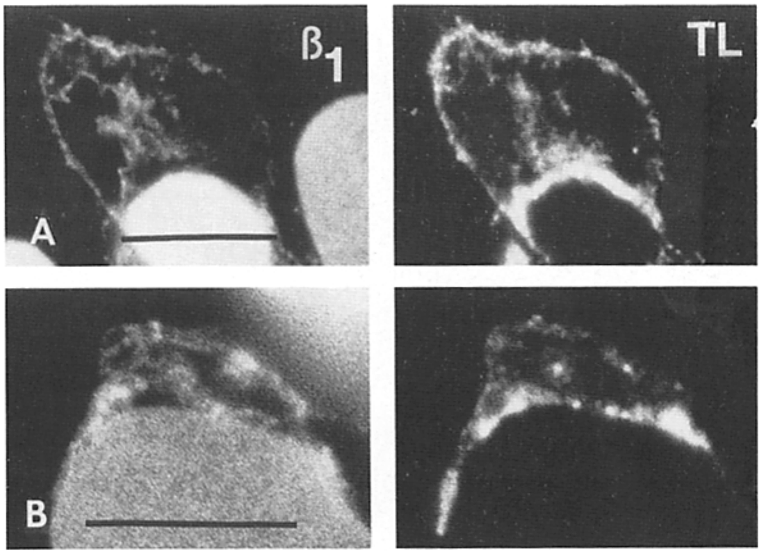

Figure 8. Aggregation of talin (*TL*) to sites of contact of cells with ES238 beads. (*A* and *B*) At left, bright labeling of the ES238 beads by fluorescein-conjugated secondary anti-rat antibody demonstrated the presence of cross-linked antibody throughout the gelatin beads, but obscured direct observation of integrin aggregation in the membrane adjacent to the beads by primary antibody ES238 label, although labeling of the membrane could be detected elsewhere in the cell. At right, talin labeling (*TL*) is intense at the membrane of the cell contacting the ES238 bead (*A*). Bars, 10 μm.

apparently produces an insoluble, multivalent FN network, such as that formed by cellular FN. This FN network allows attachment of cells and causes aggregation of the receptor. Initially, we chose RSVCEFs because these cells secrete little FN and the presence of secreted FN from normal CEFs might have complicated analysis of Con A–bead vs. FN–bead binding. Normal CEFs began to secrete detectable amounts of cellular FN after 20 min (not shown). Within the 20-min time period, however, integrin–talin coaggregation in normal cells was observed only on FN beads and not on Con A beads. Thus, integrin–talin coaggregation on FN beads may represent a general cellular response rather than a result of RSV transformation.

The aggregation of integrin at FN bead attachment sites might occur after lateral diffusion in the membrane and entrapment at the bead surface after binding FN. Alternatively, aggregation of integrin at the bead surface might occur via membrane traffic within the cell, either by a process of synthesis and exocytosis, or via endocytosis from the cell surface and recycling to sites of bead contact (McAbee and Grinnell, 1985; Molnar et al., 1987). The appearance of an intracellular pool of integrin-positive vesicles is consistent with the second possibility of membrane trafficking.

Our results demonstrate the early appearance of talin aggregation at the membrane independent of the later aggregation of α-actinin and actin. Talin aggregation may be one of the first events induced by FN binding, while α-actinin and actin become localized at cell attachment sites during the more advanced stages of cell spreading or phagocytosis. Our results also suggest that the recruitment of α-actinin and actin at later times is not FN specific because the cells attached to Con A beads showed similar results. In agreement with our studies, Grinnell and Geiger (1986) also observed in whole mounts of cells that vinculin was not detected at sites

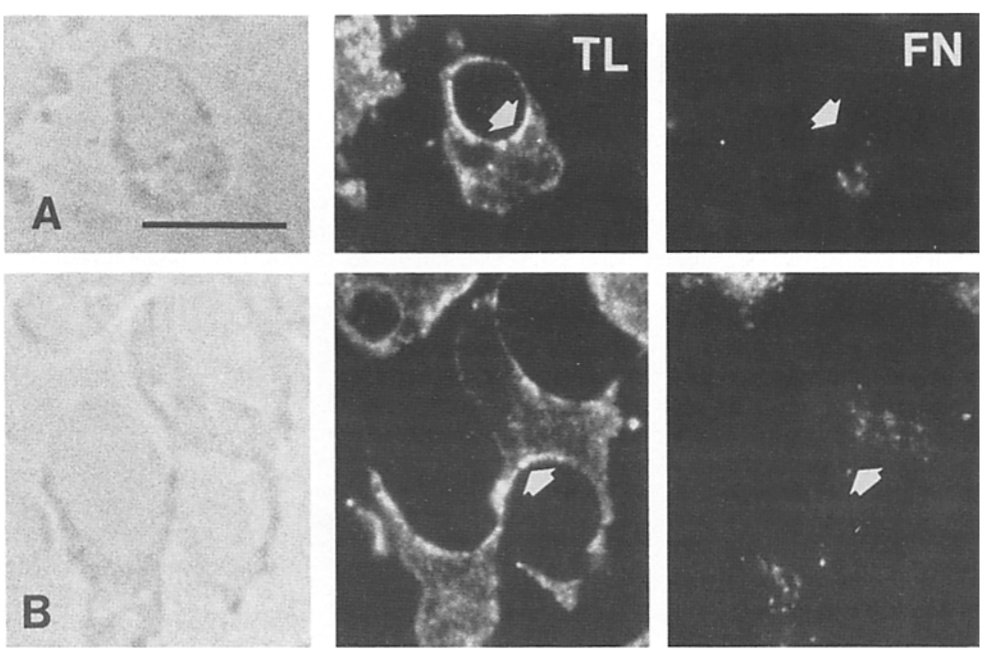

Figure 9. Talin aggregation on membranes bound to ES238 beads is not associated with secreted FN. Sections from transformed cells incubated with ES238 beads for 20 min at 22°C were double labeled for talin (*TL*) and FN. In *A* and *B*, arrows show corresponding membranes where there is talin aggregation (*TL*), but no FN label (*FN*). Intracellular FN labeling in RSVCEFs is considerably less than in normal cells. Bar, 10 μm.

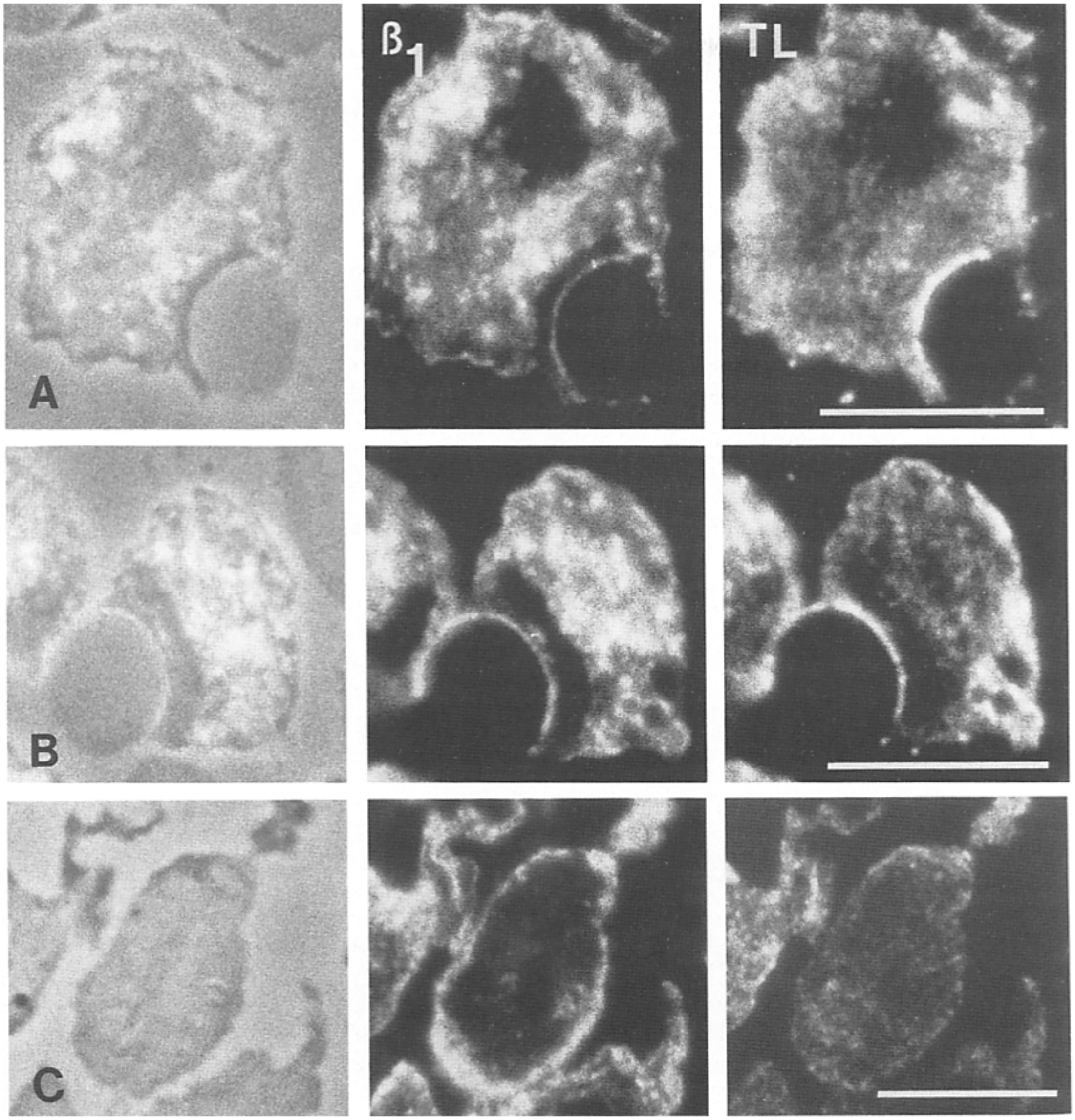

Figure 10. Aggregation of integrin (β_1) and talin (*TL*) in untransformed cells attached to FN beads. During the attachment of CEFs on FN beads for 20 min shown in *A* and *B*, integrin (β_1), observed using mAb ES238, is aggregated at the membrane in contact with the bead and a prominent internal pool of integrin-positive vesicle is apparent. Talin is aggregated adjacent to the bead but frequently does not colocalize with integrin intracellularly. (*C*) The distribution of integrin (*center*) and talin (*right*) on cells suspended in serum-free medium after trypsinization is shown. Integrin is localized at the cell surface, while talin labeling is diffuse in the cytoplasm. Note that these sections are ~1 μm in thickness and are thicker than those shown in Fig. 4. Bar, 10 μm.

of cell membranes contacting FN latex beads, whereas patches of α-actinin and actin were detected. Burn et al. (1988), however, found that in surface capping experiments α-actinin and vinculin did not associate with the capped integrin. Vinculin can apparently associate with focal contacts within 90 s of their formation in cells already in culture (DePasquale and Izzard, 1987), and focal contacts can be formed within 1–2 h in fibroblasts seeded onto planar FN substratum (Singer et al., 1987). The events described in these well-spread cells may not be comparable to the early events involving talin that we describe in our cell–bead system. We have examined the initial contact of freshly suspended cells to FN substrata before focal contact formation. In addition, our immunolabeling procedure on frozen thin sections resolves a labeling pattern which may be different from the results of other immunolabeling methods involving permeabilization of the cells which might remove cytoskeletal proteins (Grinnell and Geiger, 1986; DePasquale and Izzard, 1987; Singer et al., 1987). The commonly seen streaks of colocalized FN, integrin, talin, α-actinin, actin, and vinculin on stationary cells in culture form during the longer periods of culture used to obtain well-spread cells (Burridge, 1986; Chen et al., 1985*b*; Damsky et al., 1985). This transmembrane complex has been described earlier as the ECM contact (Chen and Singer, 1982), which may play a role in the assembly of FN fibrils and the formation of streaks where FN, integrin, and talin are colocalized (Akiyama et al., 1989; Roman et al., 1989).

We thank Yunyun Yeh and Robert Delsite for excellent technical assistance, and Lan Bo Chen and Kenneth M. Yamada for critical review of the manuscript.

This investigation was supported by U.S. Public Health Service grants R01 CA-39077 and R01 HL-33711 to W. -T. Chen.

Received for publication 3 March 1989 and in revised form 28 August 1989.

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