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Immuno-EM localization of the β_1 integrin subunit in wet-cleaved fibronectin-adherent fibroblasts

Alexandra M. L. Meijne, Diane M. Casey, Constance A. Feltkamp and Ed Roos

Division of Cell Biology, The Netherlands Cancer Institute, 121 Plesmanlaan, 1066 CX Amsterdam, The Netherlands

SUMMARY

Using immuno-EM, we have studied the distribution of the β_1 integrin subunit in chicken embryo fibroblasts allowed to adhere and spread for 3 hours on a fibronectin-coated surface in serum-free medium. The cells were wet-cleaved, which removed most of the cell body, yielding ventral plasma membranes with little, and sometimes virtually no, associated cytoskeleton.

The β_1 integrin subunit was detected with antibodies against the cytoplasmic domain. In immune fluorescence, it colocalized with adhesion plaques, in a punctate staining pattern, and often seemed to be at the periphery of the plaque. By immuno-EM, β_1 was in fact found in discrete clusters, not throughout the plaque. In deep-cleaved cells from which virtually all cytoskeleton was removed, clusters could often be seen to be located on fibronectin fibrils. Furthermore, β_1 was present in clusters at the cell margins, and isolated or in small groups at the very edge of the cell.

When fibronectin synthesis, and consequently fibril formation, was inhibited by cycloheximide, large adhesion plaque-like structures were formed at the cell margin. This phenotype was reversed by addition of soluble fibronectin, which was incorporated into fibrils. As in normal plaques, talin and vinculin were present, the plasma membrane was very close (10-20 nm) to the substratum and the fibronectin layer underneath was removed. These plaques did contain β_1 integrins but they were not in clusters.

These observations indicate that the talin-vinculin network of an adhesion plaque is normally anchored to the substratum at discrete β_1 integrin clusters that may be located on fibronectin fibrils, and that elsewhere the plaque is not necessarily attached to the substratum by interaction of integrins with matrix proteins. In the absence of fibronectin fibrils, adhesion plaque-like structures can be formed, but these are aberrant in size, location and fine structure.

Key words: adhesion plaque, focal contact, fibronectin fibril

INTRODUCTION

Cells that adhere and spread on surfaces coated with extracellular matrix proteins often form surface structures termed focal adhesions or adhesion plaques (Burridge et al., 1988; Geiger et al., 1987; Geiger, 1989). At these sites, the distance between substratum and ventral plasma membrane is very small (10-20 nm), as can be assessed by their dark image in interference reflection microscopy (IRM) and by electron microscopy of thin sections. Bundles of actin microfilaments, so-called stress fibres, terminate at the cytoplasmic surface of these adhesion plaques. The cytoplasmic part of the adhesion plaque contains many different components, but the major constituents are talin, vinculin and α-actinin (Burridge et al., 1988). We have recently shown that throughout the plaque talin and vinculin are present at a high concentration in a dense network close to the plasma membrane (Feltkamp et al., 1991).

The adhesion of cells to extracellular matrix proteins is mediated by multiple cell surface molecules, including several members of the integrin superfamily. These integrins are heterodimers, consisting of non-covalently associated α and β subunits, both of which are transmembrane glycoproteins (Hynes, 1992). In biochemical assays, the β_1 subunit or its isolated cytoplasmic domain binds to talin (Horwitz et al., 1986) and α-actinin (Otey et al., 1990), suggesting that either or both connections may provide a linkage between matrix proteins and the cytoplasmic plaque components, and thereby with the actin stress fibres.

In line with this proposed role, immune fluorescence (IF) showed β_1 integrins to be concentrated at adhesion plaques (Damsky et al., 1985; Marcantonio and Hynes, 1988; Fath et al., 1989). However, their exact location was not clear. Initially, they were seen only at the periphery (Damsky et al., 1985), in line with the observation that matrix proteins are not present under the central part of the plaque (Avnur and Geiger, 1981; Chen and Singer, 1982). However, this was later attributed to limited accessibility to the antibodies (Kelly et al., 1987). Thus, current models of the adhesion plaque (Burridge et al., 1988; Geiger et al., 1987; Geiger, 1989) are implicitly based on the assumption that the talin-vinculin network, and perhaps also α-actinin, is attached to a continuous layer of integrins.

In this study, we have used immuno-EM to localize β_1 integrins with higher spatial resolution, using antibodies

against the cytoplasmic domain (Marcantonio and Hynes, 1988). The cells were 'wet-cleaved' (Brands and Feltkamp, 1988), i.e. nitrocellulose was attached to the dorsal surface and removed. As a result, cells were cleaved at varying depths close to the ventral surface; in some cells or cell areas cleavage was so close to the surface that most of the cytoplasmic adhesion plaque components were removed, providing easy access for antibodies. We used chicken embryo fibroblasts that had spread in serum-free medium for 3 hours on a substratum coated with fibronectin. In the absence of serum vitronectin, these cells probably adhere only to fibronectin, i.e. both to the supplied substratum and the endogenously produced fibronectin. Furthermore, this adhesion is probably mainly mediated by β_1 integrins (Pytela et al., 1985; Vogel et al., 1990; Charo et al., 1990; Hynes, 1992).

We show here that adhesion plaques do not contain a continuous layer of integrins. Rather, the β_1 integrins were found at the periphery of the plaque, confirming the results of the IF studies by Damsky et al. (1985). Furthermore, the β_1 integrins were found as discrete clusters, which may be located on fibronectin fibrils. Prevention of fibril formation caused the cells to form adhesion plaques that were aberrant in size, location and fine structure.

MATERIALS AND METHODS

Cells

Chicken embryo fibroblasts (CEF), isolated from 10-day embryos, were grown in Dulbecco's modified Eagle's medium (DMEM, Paisley, UK) containing 10% fetal calf serum (FCS), 10 mM Hepes and 2 mM glutamine, and were used between passages 3 and 8.

Reagents

Immuno-purified rabbit polyclonal antibodies against talin have been described previously (Brands et al., 1990). A rabbit polyclonal antiserum against α-actinin was purchased from ICN (Costa Mesa, CA). A polyclonal antiserum against fibronectin was made by immunizing rabbits with bovine plasma fibronectin purchased from Sigma (St Louis, MO). The VIN11-5 monoclonal antibody (mAb) against vinculin, CSAT mAb against the extracellular part of chicken β_1 integrin, extravidin-FITC conjugate, bovine plasma fibronectin, laminin and cycloheximide were also from Sigma. A polyclonal rabbit antiserum raised against a peptide identical to most of the cytoplasmic domain of β_1 (Marcantonio and Hynes, 1988) was kindly provided by Dr R. O. Hynes. A similar anti-peptide serum against the α_5 integrin subunit cytoplasmic domain was a gift from Dr C. A. Buck. Rhodamine-conjugated phalloidin was from Molecular Probes (Junction City, OR). Biotinylated sheep anti-mouse IgG, donkey anti-rabbit IgG, streptavidin-peroxidase conjugate, goat anti-mouse and anti-rabbit gold-conjugated antibodies were from Amersham (UK). Protein A conjugated to 5 nm gold was prepared as described by Slot and Geuze (1985). For double-label experiments, batches were carefully screened for a low level of variation in gold particle diameter.

Immunofluorescence

CEF were harvested by trypsin-EDTA treatment and allowed to spread on fibronectin-coated coverslips for 3 hours in serum-free medium. Cells were fixed and permeabilized with methanol (5 minutes, –20°C) or fixed with 2% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 2 minutes, followed by a 15 minute treatment with 1% BSA in PBS. In some cases, the cells were wet-cleaved as described below. All immune incubations were performed at room temperature for 60 minutes, all antibodies were diluted in PBS/1% BSA. The secondary antibodies used were biotinylated and detected with extravidin-FITC. Actin filaments were visualized with rhodamine-conjugated phalloidin. FITC-labeled fibronectin was prepared as described by McKeown-Longo and Mosher (1983).

Wet-cleaving and immuno-electron microscopy

CEF were allowed to spread on fibronectin-coated Formvar/carbon-coated nickel grids for 3 hours, wet-cleaved by attachment to and removal from moist nitrocellulose as described previously (Brands and Feltkamp, 1988), and fixed with 2% paraformaldehyde for 20 minutes. After a 15 minute treatment with 20 mM glycine in PBS, the specimens were incubated with the primary antibodies for 60 minutes, rinsed in PBS, and incubated with gold-labeled Protein A (to detect polyclonal antibodies) or anti-mouse IgG (to detect mAb). For double labeling the specimens were first incubated with polyclonal antibodies, followed by 5 nm gold-conjugated Protein A, and then treated with 1% glutaraldehyde to destroy unoccupied Protein A binding sites. After treatment with 1 M glycine, the next incubation was with the second primary antibodies followed by 10 nm gold-labeled secondary antibodies. All antibodies were diluted in PBS with 1% BSA. After labeling, specimens were fixed with 1% glutaraldehyde (overnight at 4°C) and 0.1% OsO_4 (5 minutes at 4°C), stained with uranyl acetate, dehydrated in ethanol, and critical point dried. Specimens were viewed in a Philips CM10 electron microscope at 80 kV.

Cycloheximide treatment

Subconfluent monolayers were washed two times with serum-free medium and incubated for 3 hours in serum-free medium containing 50 μg/ml cycloheximide. Cells were harvested by trypsinization, washed with serum-free medium containing 100 μg/ml soybean trypsin inhibitor (Sigma) and 1 mg/ml BSA, followed by two washes in serum-free medium. Cells were allowed to spread in serum-free medium containing 50 μg/ml cycloheximide on coverslips or EM-grids coated with fibronectin. Control cells were treated similarly but in medium without cycloheximide.

Determination of distances between clusters

To measure distances between integrin clusters, an image analysis computer program was used. Clusters were defined as a set of gold particles that were less than a set distance apart from a neighbouring particle. The distance was set so that all clusters found by visual inspection were identified by the computer program. Occasionally, a single cluster was detected as two clusters at the same site on two sides of a fibril, and this was manually corrected. The centres of the clusters and the distances between these centres were then calculated.

RESULTS

Specificity of antibodies

The specificity of the antibodies against vinculin and talin was tested by western blot analysis, and was as described previously (Brands and Feltkamp, 1988; Brands et al., 1990). The monoclonal antibody against α-actinin reacted exclusively with a protein of 100 kDa (not shown). The polyclonal antiserum against β_1 integrin subunit was described by Marcantonio and Hynes (1988).

Spatial distribution of fibronectin, β_1 integrin, α-actinin and adhesion plaques

Chicken embryo fibroblasts (CEF) were allowed to spread for 3 hours on a fibronectin-coated substratum in serum-free medium. Immune fluorescence, using the CSAT mAb against

the extracellular domain of β_1, gave rise to a staining pattern consisting of thin lines (Fig. 1A). These lines coincided with adhesion plaques as defined by α-actinin accumulation (Fig. 1B) and dark spots in IRM images (Fig. 1A, inset). Occasionally, parallel stripes were seen at the edges of adhesion plaques (Fig. 1A, arrowhead), reminiscent of the 'needle eye' patterns described by Damsky et al. (1985).

This suggested that β_1 integrins were not present in the centre of the plaque. To exclude the possibility that this was due to poor access of the mAb, cells were wet-cleaved (see below) and incubated with antibodies directed against the cytoplasmic domain of the β_1 subunit. Again, needle-eyes were observed (Fig. 1C, arrowheads). Furthermore, the staining was punctate, suggesting that the β_1 integrins were located in clusters.

Fibronectin staining (Fig. 1D) revealed fibronectin fibrils located alongside adhesion plaques, which in the same figure can be seen as sites from which substratum fibronectin had been removed, as described by Avnur and Geiger (1981). Using surfaces coated with FITC-labeled substratum, we have confirmed their finding that lack of staining under the plaque was not due to poor accessibility, but caused by removal of the fibronectin coat (not shown).

Immuno-EM localization of talin, vinculin and β_1 integrin in wet-cleaved cells

As described previously (Brands and Feltkamp, 1988), wet-cleaving of cells results in removal of most of the cell body, leaving a ventral plasma membrane with associated cytoskeleton, the amount of which varies per cell and per specimen. We studied the distribution of the β_1 integrin subunit in such preparations, using antibodies against the cytoplasmic domain, in relation to the localization of the plaque components talin and vinculin. After cleaving, CEF were fixed with paraformaldehyde and incubated with antibodies. Fig. 2A shows the labeling of talin (10 nm gold) and vinculin (5 nm gold). As described previously (Feltkamp et al., 1991), these proteins were located in dense parallel and interconnected structures, probably representing adhesion plaques. Needle eye structures as indicated in Fig. 1 (length, 3-6 μm; width, up to 600 nm) would fit within the area shown in Fig. 2A (width, 600 nm).

Fig. 2B shows an area similar to that in Fig. 2A labeled with

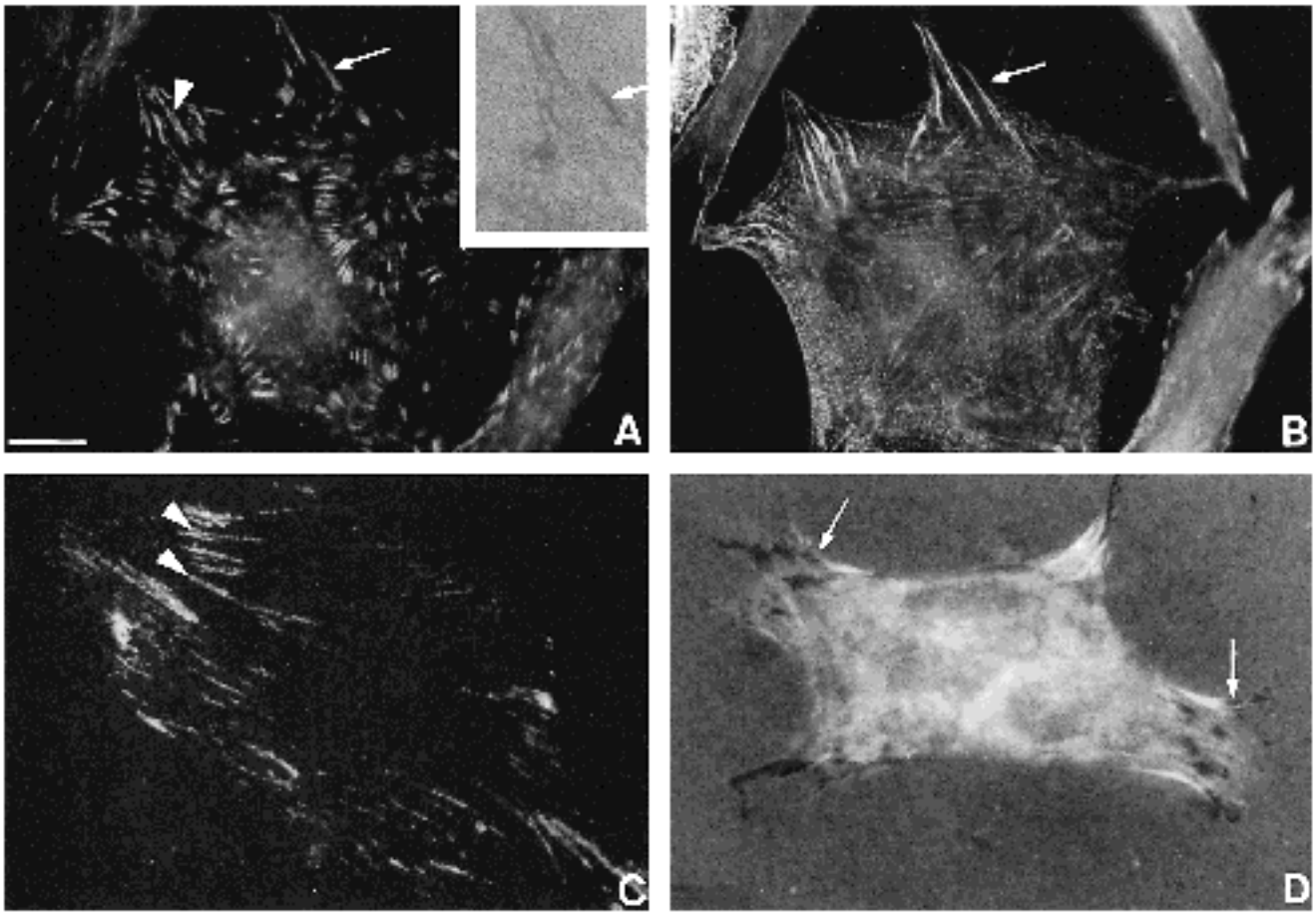


Fig. 1. CEF, spread for 3 hours on a fibronectin-coated substratum. Double-label IF of β_1 (A) detected with the CSAT mAb and α-actinin (B) stained with polyclonal antibodies after permeabilization. At adhesion plaques, visible as dark areas in IRM (A, inset), β_1 colocalizes with α-actinin (arrows). (C) When detected in wet-cleaved cells with antibodies against its cytoplasmic domain, the β_1 pattern is punctate and often in a needle-eye configuration (arrowheads). The latter can also be seen in a few adhesion plaques in permeabilized cells (A, arrowhead). (D) IF staining of fibronectin yields a strong reaction for fibronectin fibrils, often located alongside adhesion plaques, and weak labeling of the coated substratum. Under some of the adhesion plaques fibronectin is absent (arrows). Bar, 5.3 μm.

antibodies against the β_1 cytoplasmic domain: β_1 is located in clusters close to the periphery of, but does not co-localize with, the adhesion plaque material, which was similar in appearance to the talin/vinculin-labeled structures shown in Fig. 2A. The β_1 integrin was most readily detected in cells cleaved so close to the membrane that most of the overlying material had been removed. Double labeling of talin and β_1 was therefore difficult to achieve. Fig. 2C shows the results of double

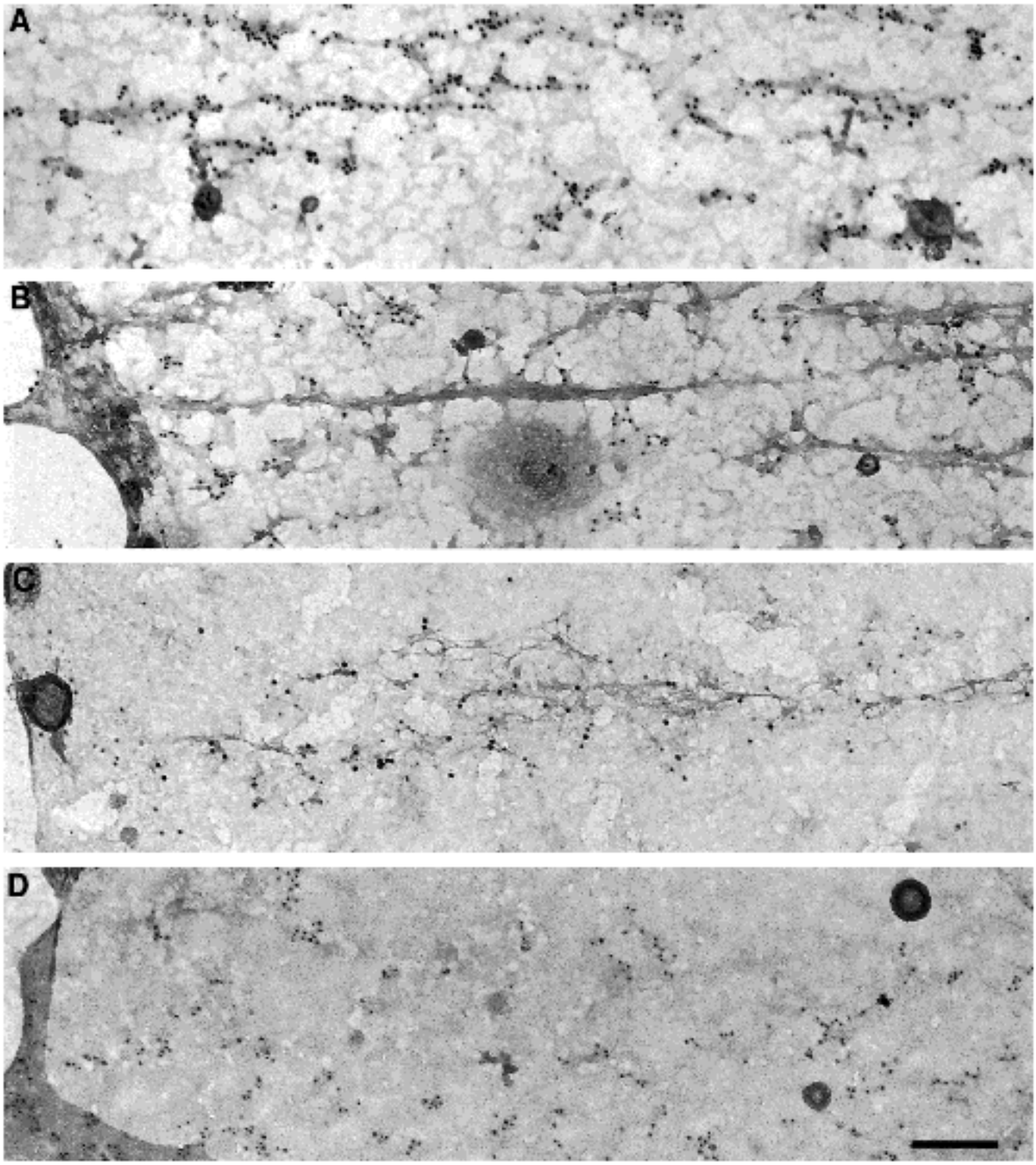


Fig. 2. EM localization of talin, vinculin and β_1 in wet-cleaved CEF. (A) Talin (10 nm gold) and vinculin (5 nm gold) are concentrated at dark bands that form part of an adhesion plaque. (B) In a similar area, β_1 is not concentrated at the bands but localized primarily at their periphery. (C) Double labeling of β_1 (5 nm gold) and talin (10 nm gold) demonstrates the difference in their localization. (D) A comparable area where all overlying material was stripped off. Again the membrane between the clusters is virtually devoid of β_1 integrin subunit. Bar, 200 nm.

labeling in an area containing only remnants of the overlying material, confirming that the majority of β_1 is located at the periphery of the area labeled by talin antibodies. Fig. 2D shows an area where all overlying material was stripped off, in a location within the cell where adhesion plaques were usually found, suggesting that even if all material that might mask the integrins is removed, the area between the integrin clusters is still virtually devoid of β_1 subunit label. Due to the absence of the characteristic overlying material, it is obviously not entirely certain that an adhesion plaque was located at this particular site. However, observation of many such areas, where several plaques should have been present, based on comparisons with similar areas in less deeply cleaved specimens, never revealed a β_1 integrin distribution comparable to vinculin and talin as shown in Fig. 2A. We therefore conclude that the absence of β_1 from the center of the plaque is not an artifact due to masking by overlying structures.

In such deep-cleaved specimens, β_1 integrin clusters could often be observed to colocalize with fibrillar structures of somewhat increased electron density (Fig. 3A). In stereo micrographs (not shown), these fibrils were seen to be located under the cell, in contrast to the few remaining cytoskeletal filaments that were present on the cytoplasmic side. In addition, β_1 clusters were seen at the cell periphery, and isolated gold particles and small groups were found at the very edges of the cell (Fig. 3B).

Identification of fibronectin fibrils

To show that the observed fibrils were composed of fibronectin, we labeled the cells with an anti-fibronectin serum, and observed them as critical-point-dried whole mounts. Fig. 4 shows fibrils that were similar in size, shape and localization to the structures colocalizing with β_1, which were heavily labeled where they extended beyond cells. Only few gold particles were present on parts of the same fibrils under the cells, probably due to reduced accessibility. Similar fibrils located on top rather than under the cell were heavily labeled throughout (Fig. 4B). To distinguish better the fibrils from the fibronectin substratum that was also labeled, and to show that the fibronectin fibrils were generated from fibronectin produced by the cells, CEF were allowed to spread on a laminin substratum in serum-free medium. These cells produced fibrils of similar morphology that reacted strongly with the anti-fibronectin antibodies, whereas the substratum remained unlabeled (Fig. 4C).

Localization of β_1 and α_5 subunits

Double labeling with antibodies against the β_1 and α_5 cytoplasmic domains, respectively, revealed both to be located in clusters on fibronectin fibrils (Fig. 5), showing that $\alpha_5\beta_1$ is at least one of the β_1 integrins located at those sites, if not the only one.

Distances between β_1 integrin clusters

Dzamba and Peters (1991) have shown that EIIIA sites in fibronectin fibrils are spaced 42 and 84 nm apart, depending on the thickness of the fibril. Presumably, the distances between the sites containing the tripeptide RGD are similar, which may thus dictate the spacing between the RGD-binding β_1 integrins. We have measured the distances between the centres of 47 β_1 integrin clusters, similar to those in Fig. 3A, on seven fibrils in three cells by computer analysis. A histogram of the measured distances shows three peaks at approximately 40, 70-80 and 110 nm (Fig. 5), consistent with the notion that clusters are located at staggered arrays of RGD sites, spaced 40 or 80 nm apart.

Role of fibronectin fibrils in the formation of adhesion plaques

Our observation that integrins are not present in the centre of the plaque, but rather on adjacent fibronectin fibrils, suggested an

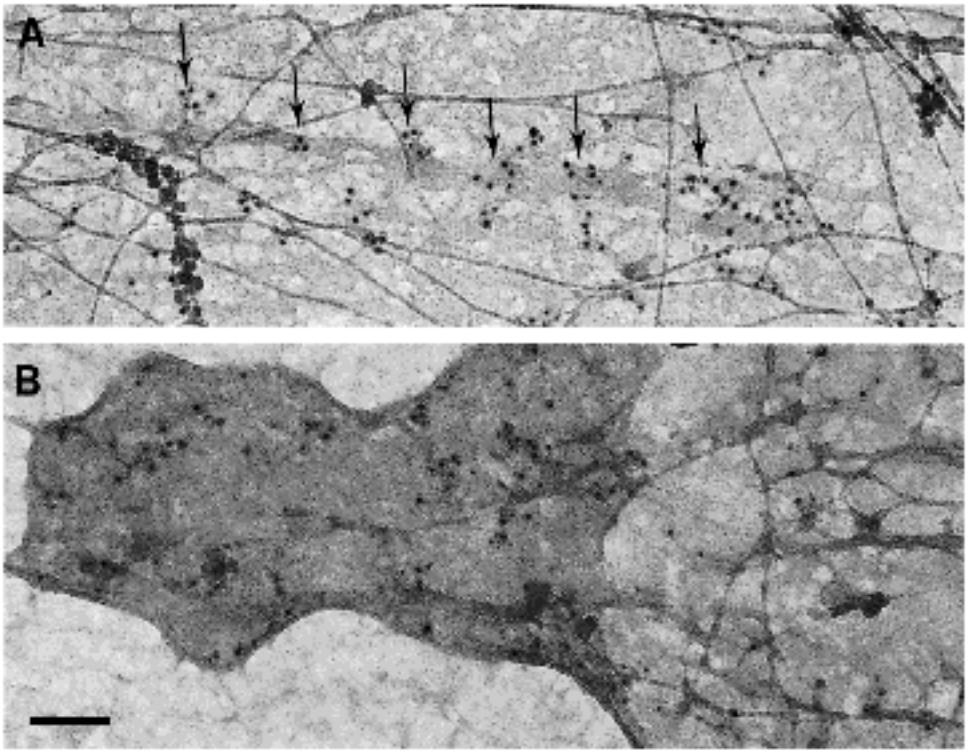


Fig. 3. High magnification of β_1 distribution in wet-cleaved CEF. (A) Most gold particles are concentrated in clusters (arrows) located over and at the side of fibrillar structures of somewhat increased electron density. (B) At the cell border, β_1 is detected by both clustered and dispersed gold particles. Bar, 100 nm.

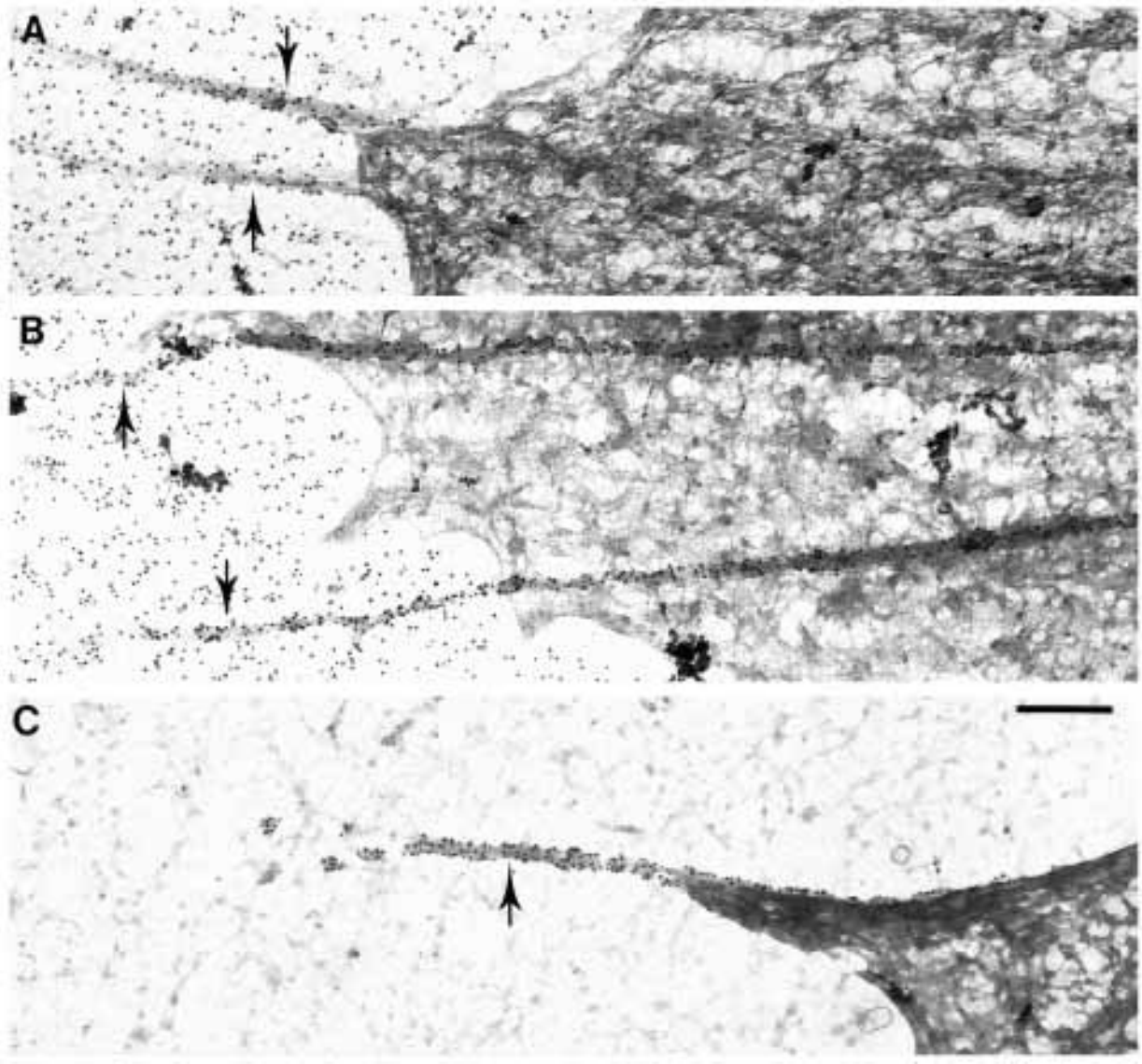


Fig. 4. Immuno-EM of fibronectin. Whole mounts of critical-point-dried CEF, spread for 3 hours on EM grids coated with fibronectin (A,B) or laminin (C). Fibrils of increased electron density beneath the ventral surface (A, only stained at the cell border), over the dorsal surface of the cells (B) and extending over the matrix (A, B, C arrows) are heavily labeled. The laminin-coated substratum is not labeled (C). Bar, 200 nm.

essential role for these fibrils in adhesion plaque formation. To investigate this, cells were treated with cycloheximide to block fibronectin synthesis for 3 hours before trypsinization and during 3 hours of spreading on fibronectin. This treatment prevented the formation of fibronectin fibrils completely (compare Fig. 7A and B). Furthermore, substratum-attached fibronectin was removed from under large marginal areas but, as compared to untreated control cells, much less from underneath the more central parts of the cell. This indicated that aberrantly large adhesion plaques had been formed only at the cell periphery. Soluble fibronectin added to cycloheximide-treated cells induced the formation of fibrils and restored normal morphology. The fibrils formed consisted of the added fibronectin as was revealed when FITC-labeled fibronectin was used (Fig. 7C). This shows that the effects observed are due to a lack of fibronectin production and not to other consequences of cycloheximide treatment.

IF double labeling of β_1 and talin, in conjunction with IRM, showed that the large marginal areas formed in the presence of cycloheximide were similar to adhesion plaques: they were black in IRM, contained talin, and β_1 was concentrated at or near these sites (Fig. 8). Wet-cleaving of cycloheximide-treated cells usually resulted in membrane preparations with very little associated cytoskeleton. Immuno-EM with talin antibodies revealed isolated gold particles and small patches, associated with filaments when these were still present (Fig. 9B). This pattern differed from the distinct dense parallel structures seen in control cells (Fig. 9A). Also the β_1 integrin distribution was affected: clusters were not seen, but quite large areas at the cell border contained a large number of gold particles

Fig. 5. In wet-cleaved cells, β_1 (5 nm gold) and α_5 (10 nm gold) integrin subunits, both detected with antibodies against their cytoplasmic domain, are colocalized in clusters. Bar, 100 nm.

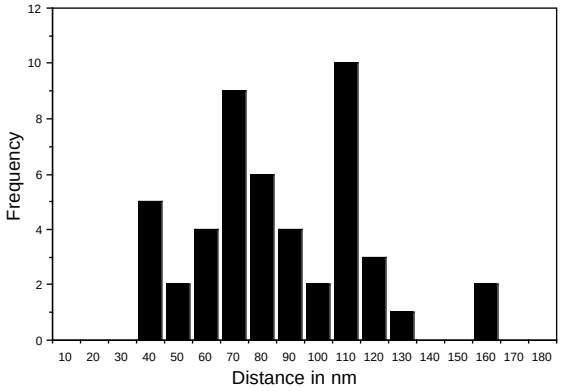


Fig. 6. Distances between β_1 clusters on and along fibrils, assessed for 47 clusters along 7 fibrils in 3 cells in micrographs comparable to Fig. 3A.

that were mainly isolated or present in small patches (Fig. 9C). Thus, these structures were not just somewhat enlarged adhesion plaques, since the fine structure and the distribution of talin and β_1 were distinctly different.

DISCUSSION

We have determined the localization of the β_1 integrin subunit by immuno-EM in chicken embryo fibroblasts that had spread on fibronectin in serum-free medium. In these circumstances, i.e. in the absence of serum vitronectin, adhesion is mediated by β_1 integrins (Hynes, 1992), probably mainly $\alpha_5\beta_1$ (Pytela et al., 1985), perhaps in conjunction with $\alpha_V\beta_3$ (Charo et al., 1990), but possibly also $\alpha_V\beta_1$ (Vogel et al., 1990). Similar localization studies reported so far were mainly performed using immune fluorescence (IF) (Damsky et al., 1985; Kelly et al., 1987; Marcantonio and Hynes, 1988; Fath et al., 1989) and revealed a pattern of stripes coinciding with adhesion plaques and fibronectin fibrils. The early studies of Damsky et al. (1985) suggested that the integrins were located at the periphery, but not in the centre of adhesion plaques in a so-called needle-eye pattern. However, this was later ascribed by Kelly et al. (1987) to the limited access of the monoclonal antibody used, since polyclonal antibodies did react with the entire adhesion plaque. Our IF results also suggest that β_1 integrins colocalize with adhesion plaques, and needle eye patterns were seen. In addition, we found a punctate staining within the stripes when using antibodies directed against the cytoplasmic domain of β_1, suggesting that the integrins were located in clusters. This pattern was not so evident in the results described by Marcantonio and Hynes (1988), who used the same antiserum. The apparently greater resolution of our observations may be due to the enhanced accessibility of the epitopes after wet-cleaving, which removes most of the dense cytoskeleton overlying the integrins.

Using immuno-EM on such wet-cleaved cells, we have previously observed that the adhesion plaque components talin and vinculin are located in electron-dense parallel strands (Feltkamp et al., 1991). In the present study, we saw substantial amounts of β_1 label only when most of this vinculin-talin complex was removed. Strikingly, the β_1 integrins were located in discrete clusters next to the remnants of the strands,

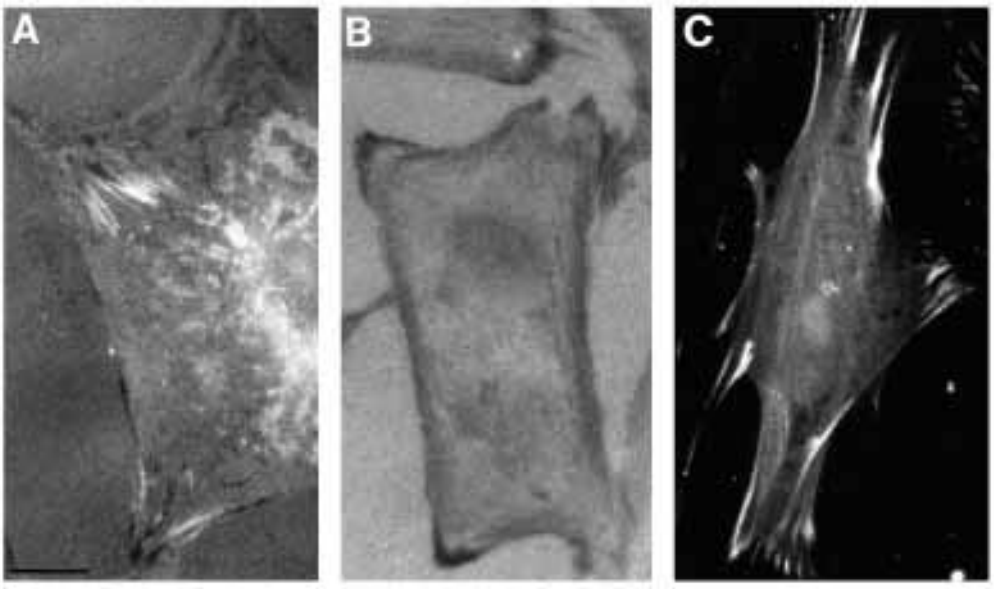


Fig. 7. Influence of cycloheximide treatment on fibronectin distribution. (A,B) IF labeling of fibronectin in CEF spread for 3 hours on fibronectin-coated matrix; (A) control cell, (B) cycloheximide-treated cell. Cycloheximide treatment inhibits formation of fibronectin fibrils, while formation of adhesion plaques under the cell body, as detected by loss of fibronectin staining, is strongly reduced. Instead, fibronectin has been removed from rather large areas at the cell border. (C) FITC-labeled fibronectin added to cycloheximide-treated cells is incorporated into fibrils. Both the distribution of the fibronectin fibrils and the shape of the cell are similar to that of control cells (Figs 1D, 7A).

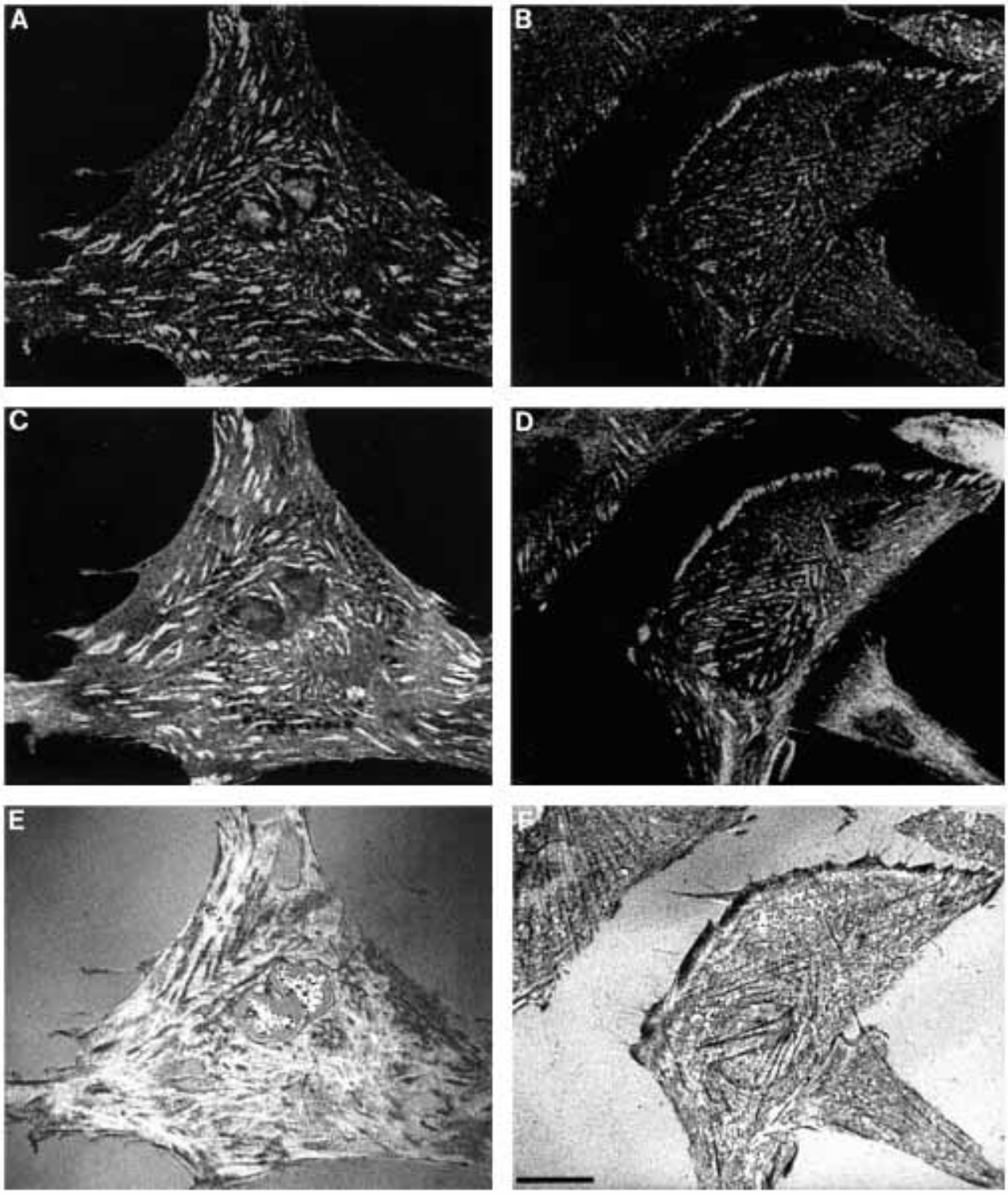
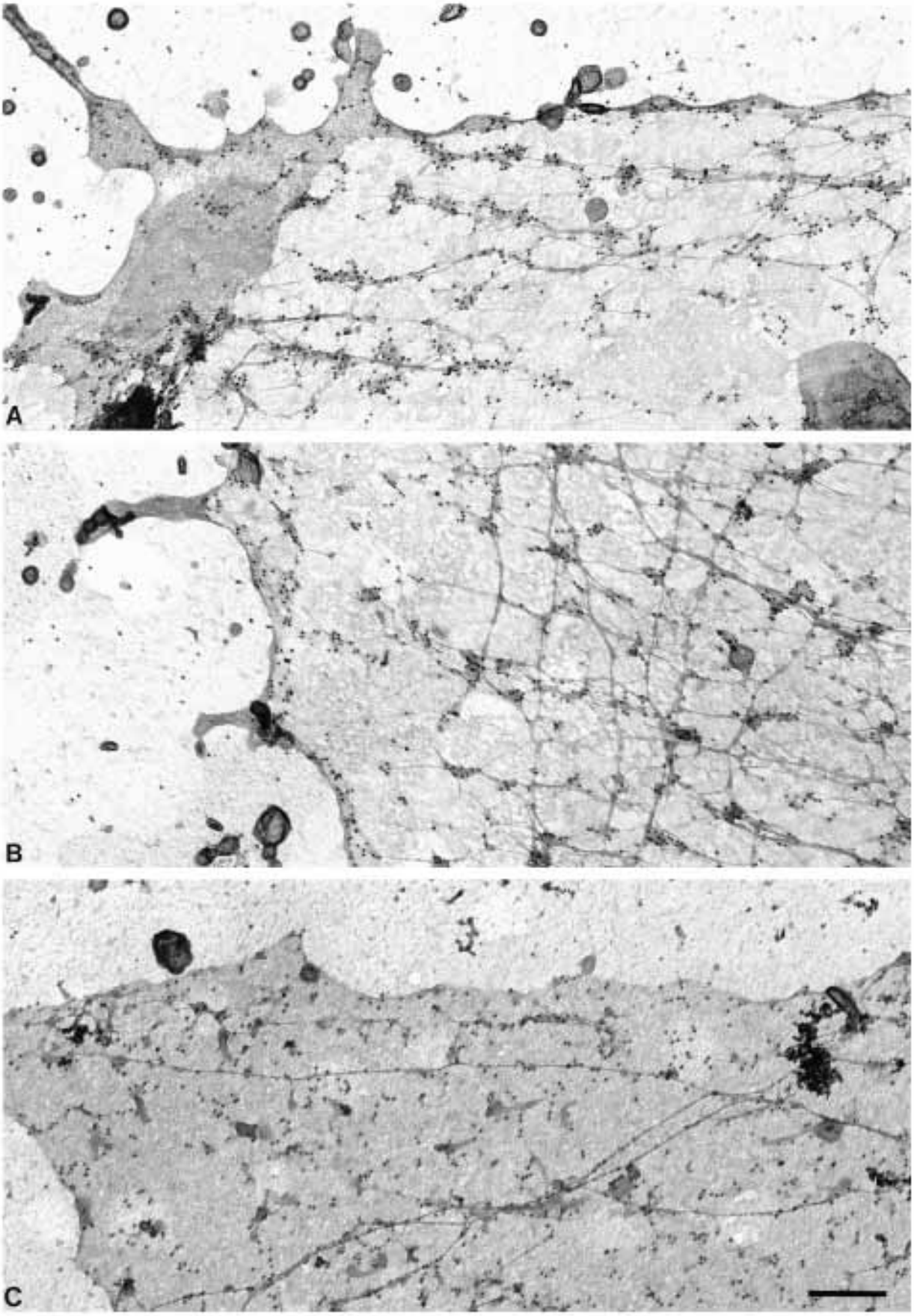


Fig. 8. Effect of cycloheximide on distribution of β_1 integrin subunit (A,B) and talin (C,D) and on IRM (E,F). (A,C,E) Control cell and (B,D,F) cycloheximide-treated cell, both spread for 3 hours on a fibronectin-coated coverslip. Note the large marginal adhesion plaques, as defined by concentrations of talin and β_1, as well as dark IRM, and the reduction in size and number of adhesion plaques in more central areas in the cycloheximide-treated cell. Bar, 5.3 μm.

Fig. 9. Immuno-EM of talin and β_1 in cycloheximide-treated wet-cleaved cells. In control cells (A), talin is concentrated over parallel structures. In cycloheximide-treated cells (B,C), parallel structures are absent or less distinct. At marginal areas, talin (B) is found both randomly dispersed and associated with remaining filaments, while β_1 (C) is detected as dispersed gold particles. The latter distribution differs from the clusters found in control cells (Figs 2B, 3A, 5). Bar, 200 nm.


A
B
C

rather than throughout the whole area. Also in similar locations in areas where all dense material had been removed, we observed no integrins between the clusters. This indicates that adhesion plaques are not built on top of a continuous layer of integrin molecules, as suggested by current models of adhesion plaques (Geiger et al., 1987; Burridge et al., 1988; Geiger, 1989). Rather, the dense talin-vinculin network (Bendori et al., 1989; Feltkamp et al., 1991) appears to be anchored at integrin clusters, which act as 'pillars' under a 'roof' made of a talin-vinculin network. The discrepancy between our results and some of the IF results reported previously is probably due to the low spatial resolution of IF, compared to immuno-EM, especially with strong signals, as are obtained with polyclonal antisera. In this respect, it is to be noted that the needle eye patterns were demonstrated most convincingly after increasing the resolution by image processing of digitized video IF images (Damsky et al., 1985). Our results support the notion that integrins are present in the periphery of plaques, but in addition show that they occur in discrete clusters. Very recently, Samuelsson et al. (1993), using a different technique, also showed that clusters of integrins are the attachment sites of actin filaments at adhesion plaques, confirming our observations.

These clusters were often found associated with fibrils underneath the cells, as could be seen in deep-cleaved cells from which virtually all the cytoskeleton had been removed. The clusters also contained the α_5 subunit, suggesting that $\alpha_5\beta_1$ is one of the fibronectin receptors located at those sites. Most parts of these fibrils under the ventral surface reacted poorly with anti-fibronectin antibodies, probably due to low accessibility, but there is little doubt that they were composed of fibronectin: close to the cell margin and where they extended beyond the cell periphery they reacted strongly with the antibodies, like similar fibrils on the dorsal surface. The distances most frequently observed between the β_1 clusters on these fibrils were approximately 40, 70-80 and 110 nm. Dzamba and Peters (1991) have shown that the EIIIA sites in fibronectin fibrils are spaced either 42 or 84 nm apart. It seems reasonable to assume that the spacing of RGD sites is similar. The distances measured are therefore consistent with the notion that the β_1 integrins cluster at staggered arrays of RGD sites in the fibrils, spaced 40 or 80 nm apart.

Our results indicate that some of the integrin clusters that anchor adhesion plaques to the substratum are located on fibronectin fibrils, which have previously been shown to be located next to, rather than under, the plaque (Birchmeier et al., 1980). This suggests an important role for fibronectin fibrils in adhesion plaque formation. To study this we inhibited fibronectin synthesis and thereby fibril formation, using cycloheximide. This had a profound effect on adhesion plaques, which was due to the lack of fibronectin synthesis and not to other effects of cycloheximide, since it was reversed by addition of soluble fibronectin, which is incorporated by cells into fibrils (McDonald, 1988; Pesciotta et al., 1990). As noted by others (e.g. see La Flamme et al., 1992), cycloheximide caused large adhesion plaque-like structures to be assembled in the margin of cells, but strongly reduced plaque formation under more central areas. The marginal structures may be considered adhesion plaques, as defined by the presence of talin and the short distance between membrane and substratum revealed by IRM. However, in contrast to the plaques in untreated cells, they do contain β_1 integrins that are diffusely spread or in small groups rather than in clusters. This shows that fibronectin fibrils are not required for the formation of adhesion plaques, but they do seem to be necessary, at least for chicken embryo fibroblasts on a fibronectin substratum in the absence of serum, for plaque formation away from the cell margins. Furthermore, the fibrils are apparently required to limit the size of a plaque to the characteristic dimensions (Burridge et al., 1988).

Both in cycloheximide-treated and in untreated cells, substratum-bound fibronectin was removed by the cells from under adhesion plaques. This was observed not only by immuno-staining, but also by using substrata coated with FITC-conjugated fibronectin, confirming the results of Avnur and Geiger (1981). Since these experiments were performed in serum-free medium, the fibronectin cannot have been replaced by vitronectin or other serum proteins. Yet the plasma membrane was close to the substratum: 10-20 nm as judged by the black IRM image. This suggests strong interaction, but it is not at all clear by which molecules this interaction is mediated. Because antibodies were not available to us, we did not exclude the possibility of the presence of $\alpha_V\beta_3$, which has been shown to bind to fibronectin (Charo et al., 1990); but even if it were located in the plaque, there are apparently no proteins underneath to which $\alpha_V\beta_3$ could bind.

Likewise, there was apparently no counterstructure for the β_1 integrins that we did observe in the large adhesion plaques in cycloheximide-treated cells, because fibronectin was not present there. However, the possibility of the presence of a small amount of fibronectin remnants, too small to be detected by IF, cannot be excluded. Another possibility is that the integrins remain associated with the previously formed vinculin-talin network, even in the absence of an extracellular ligand. This is not unlikely, since a mutant integrin not able to bind to its counterstructure (Takada et al., 1992), and chimeric proteins with a non-integrin extracellular and an integrin cytoplasmic domain (La Flamme et al., 1992; Geiger et al., 1992), still accumulate in adhesion plaques. The reason for this is probably interaction with plaque components like talin or α-actinin.

In conclusion, we have shown that β_1 integrin is not present throughout the adhesion plaque but concentrated in clusters. Furthermore, even quite large adhesion plaque-like structures can be maintained at sites where no or little integrin counterstructure is present. This suggests that other molecules than integrins participate in the attachment to the substratum.

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