a-Actinin and vinculin in human neutrophils: reorganization during adhesion and relation to the actin network

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Summary

We have studied the reorganization of vinculin and a-actinin during the process of adhesion in human neutrophils using immunofluorescence microscopy and interference reflection microscopy (IRM). Neutrophils in contact with uncoated glass formed black IRM areas in the cell periphery, indicative of very close contact with the substratum. Eight to twelve minutes after addition of cells to glass, vinculin was found to become concentrated in small patches at the cell periphery, partially colocalizing with the black IRM areas and with small F-actin-containing adherent protrusions. In contrast, vinculin was not significantly enriched in the less adherent F-actin-rich large pseudopods. a-Actinin became enriched during cell adhesion in retraction fibers and, in 40-50% of the inspected cells, also in large less adherent pseudopods where it colocalized with F-actin. The latter finding suggests a continuous dynamic reorganization of pseudopods, with incorporation of a-actinin at a certain stage.

Disruption of the actin network with cytochalasin D revealed a differential interaction of a-actinin and vinculin with the actin network. a-Actinin was strongly influenced by cytochalasin D, comparable to F-actin, and both proteins formed colocalizing peripheral caps in 10^-5 M of the drug. Vinculin organization in contrast was not affected by up to 10^-6 M cytochalasin. At 10^-5 M of the drug, however, the patches disappeared completely, vinculin now assuming a diffuse cytoplasmic location. Our results suggest a specialized function of vinculin in adhesion sites of human neutrophils, whereas a-actinin may structure the actin network in retraction fibers and in less adherent pseudopods.

Key words: neutrophils, a-actinin, vinculin, actin.

Introduction

Neutrophils, in order to destroy pathogens efficiently in tissue lesions, must adhere to the capillary endothelium, penetrate the basement membrane and migrate through the connective tissue to the source of infection. Clearly, a functional actin network, serving as a force-generating mechanism, is instrumental in these locomotory and adhesive functions. Indeed, chemotactic activation of neutrophils is accompanied by a dramatic reorganization of actin filaments: the amount of polymerized actin is rapidly increased, and pseudopods enriched in F-actin are formed (Zigmond, 1989). In locomoting cells, these F-actin-rich protrusions and membrane ruffles are especially formed at the front, where a continuous polymerization of actin is thought to occur (Small, 1989; Theriot and Mitchison, 1991). A variety of actin-crosslinking and bundling proteins, among them a-actinin, may stabilize the actin network in these lamellipodia (Lazarides, 1976; Small, 1989). The role of such crosslinking proteins in cell locomotion is, however, unclear, as a Dictyostelium mutant lacking more than 99% of its a-actinin has shown normal motility (Schleicher et al., 1988).

Cell locomotion in addition depends on a reversible, transient contact of the cell with substratum, for instance at the base of the lamellipodium and at the contracted tail (Chen, 1981; Small, 1989). An interaction of actin filaments with an adhesion complex bound to the substratum, and application of force to this site by rearward movement of the cytoskeleton, has been proposed to result in forward movement of motile cells (Kucik et al., 1991). Little is yet known about the molecular mechanism of neutrophil to substratum adhesion, and on the putative proteins involved in this process. Certainly, a functional actin network is necessary. For instance, adhesion of neutrophils to plastic has been shown to result in a two- to three-fold increase in F-actin (Southwick et al., 1989). Conversely, cytochalasin, which blocks actin assembly at the barbed ends (Cooper, 1987), inhibited both the adhesion-dependent rise in F-actin, and spreading of the cells (Southwick et al., 1989). Integrins, receptors for extracellular matrix proteins, have been implicated in
neutrophil adhesion and locomotion, as antibodies directed against leucocyte-specific integrins have been shown to interfere with these processes (Larson and Springer, 1990). Integrins are thought to connect with their cytoplasmic domain to the actin network via a chain of proteins (Burridge et al., 1988; Geiger, 1989). The molecular mechanism of this linkage has not yet been clarified for motile cells. Such a linkage, however, appears to exist, as for instance, retention of a laminin receptor in the cytoskeletal framework has been shown to occur upon adhesion of macrophages to laminin (Shaw et al., 1990).

We have recently identified two actin-associated proteins, vinculin and α-actinin, in human neutrophils (Niggli and Jenni, 1989). These proteins have been implicated to mediate actin to integrin linkage in adhesion sites (focal contacts) of stationary cells such as fibroblasts, together with other proteins (Burridge et al., 1988; Geiger, 1989). We could moreover demonstrate that chemotactic peptide promoted a significantly increased association of α-actinin (but not of vinculin) with the Triton X-100-insoluble cytoskeleton (Niggli and Jenni, 1989). α-Actinin could thus play an important role in structuring the actin network in these motile cells. Vinculin appears to interact less strongly with the actin filaments, in a manner disrupted by the extraction conditions.

In order to obtain more information on the role of α-actinin and vinculin in neutrophil motility and adhesion, we have studied the dynamic reorganization of these proteins during the process of cell adhesion in human neutrophils, using indirect immunofluorescence microscopy. Moreover, we have compared the locations of these proteins with that of F-actin and with areas of the cell surface, which are in very close contact with the substratum. These areas were visualized with interference reflection microscopy (IRM)1, whereby, for example, focal contacts of fibroblasts appear black (Izzard and Lochner, 1976). Our results show that vinculin, during the process of neutrophil adhesion, assumes a peripheral distribution in areas of strong adhesion, whereas α-actinin is more prominent in F-actin-rich pseudopods and retraction fibers. Studies on cytchalasin D revealed a differential interaction of these two proteins with the actin filaments.

**Antibodies**

The monoclonal anti-α-actinin antibody, clone BM-75.2, has been obtained both from Serotec (Kidlington, UK) and from Biomakor (Rehovot, Israel). This antibody has been shown to react specifically with a band comigrating with purified chicken gizzard α-actinin on transblots of total human neutrophil proteins (Niggli and Jenni, 1989). The monoclonal anti-vinculin antibody, clone vin-11-5, raised against chicken gizzard vinculin, was obtained from Biococcus (Rehovot, Israel). It reacted exclusively with a band comigrating with chicken gizzard vinculin on transblots of total human neutrophil proteins (not shown). A polyclonal antibody raised in rabbits against non-muscle actin was a kind gift from Dr. C. Chaponnier (University of Geneva, Switzerland). Fluorescein-conjugated goat anti-mouse and anti-rabbit gamma globulins (highly fluorescent) were obtained from Antibodies Incorporated (Davis, CA).

**Neutrophil isolation**

Neutrophils were isolated from heparinized (10 units/ml) human blood obtained by venepuncture from healthy volunteers. Leukocytes were separated from erythrocytes with Isopaque-Methocel (Boyum, 1968), followed by a discontinuous Hypaque-Ficoll gradient (Ferrante and Thong, 1980). Isolated neutrophils were washed several times in basic medium containing 1% HSA, followed by several washes in medium containing 0.2% HSA. The cells were finally resuspended in medium without HSA. The resulting preparation contained 95-97% neutrophils with few contaminating lymphocytes and platelets.

**Fluorescence microscopy**

Neutrophils in 450 μl basic medium (0.5 × 10^6 cells/ml) were placed on glass coverslips mounted in Sykes-Moore chambers, together with 10^−9 M [NLPNTL, final concentration (added in 50 μl basic medium), followed by incubation at 37°C for the times specified in the figure legends. Cells were subsequently fixed with 3.7% paraformaldehyde for 10 minutes at 37°C, and were afterwards washed several times with PBS, pH 7.4. Paraformaldehyde was added as a 7.4% stock solution in 25 mM MES, pH 7 (500 μl) to cells in 250 μl basic medium. Neutrophils were permeabilized with 1% Triton X-100 in PBS for 1 minute at room temperature and were washed again with PBS. In order to block unspecific staining, cells were incubated for one hour at 37°C with a 0.1% normal goat serum in PBS containing 0.02% NaN3. The samples were subsequently incubated with the first antibody (monoclonal anti-vinculin or monoclonal anti-α-actinin, both diluted 1:50 in PBS containing 10% normal goat serum and 0.02% NaN3, or polyclonal anti-actin antibody, diluted 1:100) for 45 minutes at 37°C. After washing the cells several times with PBS, the appropriate fluorescein-conjugated second antibody was added, diluted 1:100 in PBS containing 10% normal goat serum. Incubation with the second antibody was for 35 minutes at 37°C. The samples were then washed with PBS and embedded in a solution containing 50 mM Tris-HCl, pH 8.4, 40% (v/v) glycerol and 1 mg/ml diazabicyclooctan. For double-labeling experiments the cells were first fixed and stained for α-actinin or vinculin as described above. The cells were subsequently washed several times with PBS, followed by incubation with 0.5 i.u./ml rhodamine-phalloidin (final concentration) diluted in PBS containing 0.05% HSA for 10 minutes at 37°C. The reaction was stopped by incubation of the cells with PBS containing 4% HSA for 3 minutes at room temperature. Subsequently, they were washed and embedded as described above. For fluorescence, the cells were examined with an epifluorescence microscope.
and photographed using a 100×/1.30 objective (Plan-Neofluor) on an Axiovert 35 microscope from Zeiss (film: Kodak Tmax 400). Filter combinations BP 450-490/FT 510/LP 520 and BP 545/FT 580/LP 590 from Zeiss were used to detect fluorescein-conjugated antibodies and rhodamine-phalloidin. In addition, filter KP 560 from Zeiss was used in double-labeling experiments for the detection of fluorescein-conjugated antibodies, to eliminate emission above 560 nm. For IRM, a Zeiss Axiovert 30 microscope was used with a 63×/1.25 N.A. objective (antiflex neofluor), equipped with the Zeiss IRM system.

Cytochalasin D treatment of neutrophils

Cells (0.5×10⁶ cells/ml, 450 µl per assay) were incubated on glass coverslips for 30 minutes at 37°C with 10⁻⁷ M fNLPNTL, final concentration, added in 50 µl basic medium, and in the presence of different concentrations of cytochalasin D, as indicated in the figure legends. The neutrophils were subsequently fixed, permeabilized and labeled for α-actinin and F-actin or vinculin, as described above.

Results

Formation of cell-substratum contacts

Human neutrophils adhering to glass coverslips in the absence of protein appeared to form very close contacts 4-10 min after start of the incubation, as visualized with the IRM technique in living cells photographed after different time intervals upon addition to glass coverslips (Fig. 1). Peripheral black areas indicating very close apposition of the plasma membrane to the substratum were expressed mostly asymmetrically at the more contracted part of the cells, where retraction fibers are formed. Few cells expressed black areas all around the cell periphery. The central part of the cells was of a lighter grey. As shown in Fig. 1, the cells moved very little. They demonstrated some shape changes, but did not locomote. The black adhesion sites were also quite stable, although small changes in shape and size of these sites occurred continuously (see Fig. 1, 4 to 30 minutes). The cells thus express, even under conditions of low motility due to strong adhesion on uncoated glass, asymmetric formation of adhesion sites. Note the cell indicated with arrowheads in Fig. 1: at 6 minutes it has expressed black areas at both sides; at 8 minutes the adhesion site at the right has disappeared, and the left one now remains stable until at least 30 minutes after the start of adhesion.

Reorganization of F-actin, α-actinin and vinculin during the process of cell adhesion

The expression of peripheral black IRM areas during neutrophil adhesion was compared with the time-dependent reorganization of cytoskeletal proteins. A representative experiment is shown in Fig. 2. The sequence of events was very reproducible for different cell preparations, although some variations occurred in the velocity of the changes. Neutrophils were incubated on glass coverslips in the presence of the chemotactic peptide fNLPNTL (10⁻⁷ M) at 37°C for different times, followed by fixation with formaldehyde and staining for the different proteins. Isolated neutrophils, incubated at 37°C in suspension in the absence of stimuli in plastic tubes, exhibited a round morphology, with diffuse cytosolic location of F-actin as visualized by staining with rhodamine-phalloidin (not shown; see Keller et al., 1990). Incubation on glass instead of plastic induced a variety of changes in cell morphology and cytoskeletal organization. After four minutes on glass, the cells were still partially round, with thin black IRM areas appearing in the cell periphery (Fig. 2 a). Note that, due to repeated washing of the cells after fixation on the glass coverslips (see Materials and methods), cells not firmly adhering to the glass were removed. In Fig. 2, we thus observed only cells that had made a contact with glass strong enough to withstand the washing process. F-actin accumulated at the early stage of cell adhesion mainly in peripheral areas, which appeared grey in IRM (arrows in Fig. 2 A and a). The peripheral thin black IRM areas in contrast contained hardly any detectable F-actin (arrowheads in Fig. 2 A and a). Eight to sixteen minutes later, the cells became more elongated and developed larger black areas and finally retraction fibers. The peripheral adhesion sites showed spiky extensions corresponding to these retraction fibers. F-actin now accumulated also in the adherent part of the cell; in small protrusions and retraction fibers (arrowheads in Fig. 2 B,b,c,c). The F-actin-rich large pseudopods always corresponded to grey, never to black, IRM areas (arrows in Fig. 2 B,b,c,c), and extended over these areas into the light background. This is particularly evident for the two lower cells in Fig. 2 C,c. 60-90% of the cells showed such an asymmetric distribution. A polyclonal anti-actin antibody gave a comparable location (not shown). F-actin is thus differently organized in the different parts of the cell, possibly correlated with the local state of adhesion.

α-Actinin showed, four minutes after addition of the cells to glass, mainly a diffuse cytoplasmic staining with some concentration in parts of the cell periphery (Fig. 2 D and d). During the next four minutes, α-actinin accumulated in peripheral patches, which partially colocalized with dark IRM areas (arrowheads in Fig. 2 E and e), at the same time at which F-actin also appears in the black IRM sites. Some variable cytoplasmic staining also occurred. Later, α-actinin was found to be more diffusely distributed in the adherent part of the cell, with a punctuate staining in the cell periphery (arrowheads in Fig. 2 F and f).

Vinculin, at four minutes after addition of cells to the coverslips, showed a continuous cortical location and less intense cytoplasmic staining (Fig. 2 G and g), suggesting an association with the plasma membrane. Eight to sixteen minutes later, the protein showed a patchy staining at the cell periphery, which at least partly coincided with black IRM areas (arrowheads in Fig. 2 H,h,i,i). Vinculin was not uniformly distributed over the black IRM areas. Rather, the protein was enriched in patches located in these areas, and also appeared, although less frequently, in the periphery of grey cell areas. In addition to cell adhesion, an unknown factor therefore appears to determine its
Fig. 1. Time course of neutrophil adhesion to glass studied by IRM. Cells in basic medium were placed on glass coverslips at 37°C and were observed under a Zeiss microscope equipped for IRM at the times indicated in minutes on the different panels. Time 0 minutes corresponds to the time of addition of the cells on the glass coverslips. Cells exhibited black areas indicating very close contact with glass, mainly asymmetrically at one end. Note, however, the cell at 6 minutes, which first expressed two adhesion sites in a non-polar fashion (arrowheads), and at later times (8') showed just one site (arrowhead). Bar, 19 μm.

During adhesion, formation of peripheral black IRM areas thus is accompanied by the accumulation of α-actinin and vinculin into part of these areas. F-actin accumulates first mainly in less adhesive areas, and peripheral location. Incubation with control ascites fluid instead of the specific antibodies to vinculin or α-actinin resulted in very faint diffuse staining in the center of the cells (not shown).
appears subsequently, at the same time as α-actinin and vinculin, also in the adhesive part of the cell.

**Differential association of α-actinin and vinculin with F-actin in neutrophils**

In double-labeling experiments we have attempted to obtain more precise information on the relative localization of α-actinin and vinculin in the adherent neutrophil. In Figs 3 and 4, the localization of vinculin and α-actinin was compared with that of F-actin, and with sites appearing very dark in IRM. As shown in Fig. 3, the peripheral vinculin patches colocalized with part of the F-actin-containing retraction fibers and with adherent small F-actin-containing protrusions around the cell body (arrowheads). Most of the vinculin patches or streaks corresponded to dark grey or black IRM areas, whereas parts of the dark areas were free of vinculin (compare Fig. 3 A' and a; lower cell on the right). A diffuse fluorescence was observed in the less adherent part of the cell, but no obvious enrichment of vinculin in less or non-adherent F-actin-rich pseudopods could be detected (arrows in Fig. 3 A,A',a). Some cells expressed on one side retraction fibers, and on the other side a cell margin appearing black in IRM. This margin did not stain very strongly for F-actin, but contained vinculin (arrowheads in Fig. 3 A,A',a, lower cell on the left). This may represent a transient state, whereby motile pseudopods attach to the substratum, thereby accumulating vinculin. Only at a later stage would retraction fibers be formed.

As shown in Fig. 4, α-actinin colocalized with F-actin in neutrophils, in the less adherent large pseudopods and cell margins (arrows), and also near and in retraction fibers (arrowheads). Such a colocalization in pseudopods was found in 53 ± 2% of cells expressing F-actin-rich pseudopods (mean ± s.d. of 3 experiments; 150-300 cells inspected per experiment). This number corresponds to 45 ± 4% of total cells. In the remaining cells, α-actinin staining was restricted to retraction fibers, where it clearly colocalized with F-actin and was not visible in pseudopods. Examples for the latter distribution are shown in Fig. 5 A,A'. This protein may thus be involved in structuring the actin network in retraction fibers, in cytosolic locations and, transiently, also in F-actin-rich pseudopods. Note the fine linear F-actin structures visible in the cytosol of the cell on the right, Fig. 4 B, or the lower cell on the left in Fig. 4 A. They may correspond to thin actin bundles. The results shown on the localization of the different cytoskeletal proteins have been obtained in the presence of the chemotactic peptide fNLPNTL (10⁻⁹ M). Omission of the peptide did not affect the results (data not shown).

**The effect of cytochalasin D on the location of cytoskeletal proteins in neutrophils**

In order to obtain additional information on the interaction of α-actinin and vinculin with the F-actin network, their susceptibility to cytochalasin D, which disrupts actin networks (Cooper, 1987), was studied. Neutrophils were incubated together with different concentrations of cytochalasin D and 10⁻⁹ M fNLPNTL for 30 min at 37°C on glass coverslips, followed by fixation and staining. Fig. 5 A,A',a, shows control cells incubated in the absence of cytochalasin D, double-labeled for F-actin and α-actinin, and, in a separate experiment, for vinculin. Cells were well spread, with F-actin located in the pseudopods and retraction fibers, and α-actinin enriched mainly in the latter structures (arrowheads in Fig. 5 A and A'). As shown above, vinculin was present in peripheral patches, as well as showing some diffuse cytoplasmic staining (Fig. 5 a). In the presence of 10⁻⁷ M cytochalasin D no marked changes occurred, either in cell morphology or in protein localization (Fig. 5 B,B',b). Incubation with 10⁻⁶ M cytochalasin D led to the cells rounding off from the glass coverslip, accompanied by a striking re-arrangement of F-actin and α-actinin. The retraction fibers and pseudopods disappeared. F-actin and α-actinin both accumulated in colocalizing peripheral aggregates or caps (arrowheads in Fig. 5 C and C'). Small F-actin-containing cell protrusions were still visible. The peripheral vinculin patches, in contrast, appeared to be unaffected by these concentrations of cytochalasin D (Fig. 5 c). In the presence of 10⁻⁵ M cytochalasin D the cells had rounded off from the substratum, although they were still adhering to the glass (see also Southwick et al., 1989). F-actin and α-actinin were almost exclusively located in colocalizing peripheral aggregates or caps (arrowheads in Fig. 5 D and D'). Vinculin was also affected, albeit in a different manner, by this concentration of cytochalasin D: the peripheral vinculin patches disappeared completely, the protein now showing a diffuse cytoplasmic staining (Fig. 5 d).

The above findings reveal a differential interaction of α-actinin and vinculin with the F-actin network. α-Actinin appears to relate very closely to the actin filaments, before and after cytochalasin D treatment. The drastic rearrangement of the actin network is accompanied by a corresponding change in α-actinin location. Vinculin appears to be more resistant to cytochalasin D treatment. Its localization in the peripheral patches may be determined by factors additional to actin network integrity. Nevertheless, the results suggest an indirect or direct connection between vinculin and the actin network, as higher concentrations of cytochalasin D induce redistribution of vinculin from the peripheral patches to a diffuse cytoplasmic location, unrelated to F-actin.

**Discussion**

**Contact formation and concomitant redistribution of cytoskeletal proteins during neutrophil adhesion**

We have placed human neutrophils on glass coverslips in the presence of divalent cations and in the absence of protein. We have studied the development of contacts between cells and glass, and the reorganization of vinculin, α-actinin and F-actin during adhesion. We observed that approximately five minutes after addition to glass, the cells formed very close contacts appearing
Fig. 2. Time-dependent reorganization of F-actin, α-actinin and vinculin during neutrophil adhesion to glass. Cells in basic medium were placed on glass coverslips at 37°C in the presence of 10^{-9} M fNLPNTL, and were fixed after incubation for 4 minutes (A,a,D,d,G,g), 8-12 minutes (B,b,E,e,H,h) and 16 minutes (C,c,F,f,I,i) at 37°C. The cells were then permeabilized and labeled for F-actin with rhodamine-phalloidin (A,B,C), or with anti-α-actinin (D,E,F) or anti-vinculin (G,H,I) antibodies. Corresponding IRM images are also shown (a-i). F-actin was detectable, during the first minutes of cell adhesion, first at the cell periphery appearing grey in IRM (arrows in A,a,B,b,C,c), and only later was it detectable also in black IRM areas (arrowheads). α-Actinin and vinculin accumulated during the first 8-12 minutes in peripheral areas partially colocalizing with these black sites (arrowheads in E,e,F,f, H,h,I,i), together with F-actin. Bar, 16 μm.

The formation of these dark IRM sites during cell adhesion correlated with the appearance of vinculin in small patches at the cell periphery, which often colocalized with the contact sites (Fig. 2). α-Actinin also partly colocalized with the black IRM sites, especially in the earlier phase of adhesion (approximately 10 minutes after addition to glass). At longer times of incubation mainly vinculin persisted in the adhesion sites.

The vinculin-containing adhesion sites appear to be different from “podosomes”, special adhesions observed in virus-transformed cells and in cells of monocytic origin (Marchisio et al., 1987). “Podosomes” are enriched in vinculin, α-actinin, talin and F-actin. They occur not only in the cell periphery, but also in the center of the cell, whereas the vinculin-containing neutrophil adhesions described here are restricted to the cell margin. Moreover, the shape of the latter is elongated, not dot-like as that of the “podosomes”.

The vinculin-containing neutrophil adhesions also differ from focal contacts of fibroblasts, as they do not correspond to anchorage points for stress fibers. We did not observe stress fibers as a main feature in adherent neutrophils (see also Malech et al., 1977) although thin actin bundles, not obviously associated with adhesion sites, were just detectable in some cells (Fig. 4). The neutrophil adhesion sites rather corresponded to the end of F-actin-containing small cell protrusions and retraction fibers (Figs 2-4).

The finding that vinculin is detectable only in part of the adhesion areas (Figs 2,3) suggests local differences in the structure of these areas, which may be generated
Fig. 3. Comparison of the localization of vinculin with that of F-actin and with the corresponding IRM images. Cells were incubated for 16 minutes at 37°C on glass coverslips in the presence of 10⁻⁸ M fNLPNTL, fixed, permeabilized and double-labeled for F-actin with rhodamine-phalloidin (A,B) and for vinculin with a specific antibody (A',B'). The corresponding IRM images are also shown (a,b). The peripheral vinculin patches showed a partial colocalization with black IRM areas and small F-actin-containing protrusions (arrowheads). F-actin-rich pseudopods appearing grey in IRM did not show a striking enrichment of vinculin (arrows in A,A',a). Bar, 17 μm.

during dynamic remodelling of these sites in motile cells. In fibroblasts the intensity of focal contact staining for α-actinin also varies within a single cell (Pavalko and Burridge, 1991). The authors therefore propose multiple modes of actin filament to membrane attachment, whereby attachments involving different proteins may reflect different stages in focal contact formation. Indeed different modes of actin to integrin linkage have been proposed. Vinculin may for example interact directly with actin (Westmeyer et al., 1990), or α-actinin with integrins (Otey et al., 1990).

The rearrangements of cytoskeletal proteins during cell adhesion were independent of the presence or absence of the chemotactic peptide fNLPNTL (not shown). They appear thus mainly to be induced by the contact of the neutrophils with glass. Interestingly, massive accumulation of F-actin was observed first in the less adherent parts of the cell (Fig. 2). According to Southwick et al. (1989), adhesion of neutrophils to plastic induces not only a rearrangement, but also an increase in the actual amount of F-actin. The adhesion-dependent stimulus apparently is generated through a
pertussis toxin-insensitive pathway and involves an increase in cytosolic Ca\(^{2+}\). Our findings suggest that increased actin polymerization may occur first in the non-adherent part of the cell, or alternatively that locally formed F-actin-rich pseudopods immediately detach from the substratum upon formation.

**Nature of the adhesion sites**

The interpretation of IRM images is not always unambiguous. For instance, very dark fringes often seen at the margin of spread, flattened cells could be caused by interference with reflections at the upper membrane (Gingell, 1981), and are not necessarily indicative of very close contacts. The veil-like neutrophil protrusions that appear very dark in IRM, but do not stain for F-actin or vinculin (see Fig. 3), could indeed correspond to very thin, not strongly adherent cell protrusions. Moreover, black areas may in some situations turn into grey by reflecting structures in the cytoplasm (Keller et al., 1983). We think, however, that the often asymmetric expression of black IRM sites in the periphery of well-spread neutrophils represents

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**Fig. 4.** Comparison of the localization of α-actinin with that of F-actin and with the corresponding IRM images. Cells were incubated for 16 minutes at 37°C on glass coverslips in the presence of 10^{-9} M L-NLPNTL, fixed, permeabilized and double-labeled for F-actin with rhodamine-phalloidin (A,B) and for α-actinin with a specific antibody (A',B'). Corresponding IRM pictures are also shown (a,b). α-Actinin colocalized with F-actin in F-actin-rich pseudopods appearing grey in IRM (arrows) and also in cytosolic locations near, and in retraction fibers, (arrowheads). Bar, 17 μm.
Fig. 5. The effect of different concentrations of cytochalasin D on the organization of vinculin, α-actinin and F-actin in neutrophils adhering to glass. Cells were incubated either without cytochalasin D (A,A',a), or with $10^{-7}$ M (B,B',b), $10^{-6}$ M (C,C',c) or $10^{-5}$ M (D,D',d) of cytochalasin D, and always in the presence of $10^{-9}$ M fNLPNTL, for 30 minutes at 37°C on glass coverslips. The cells were subsequently fixed and either double-labeled for F-actin with rhodamine-phalloidin (A,B,C,D) and for α-actinin with a specific antibody (A',B',C',D'), or stained for vinculin (a,b,c,d). In cells incubated with no or low concentrations of cytochalasin D, α-actinin colocalized with F-actin in retraction fibers (arrowheads in A,A',B,B'), whereas vinculin was concentrated in peripheral patches (a,b). Higher concentrations of cytochalasin D induced accumulation of F-actin and α-actinin into corresponding peripheral caps (arrowheads in C,C',D,D'). Vinculin, in contrast, was shifted from the patches to a diffuse cytoplasmic location (d). Bar, 15 μm.
Network disruption is thought to result mainly from the Mullins (1987). Cytochalasin D-induced foci in PtKx cells (Wolf and coordinated reorganization of F-actin and α-actinin, M, induced rounding up of the cells, accompanied by a filament-severing activity of the drug. Foci formation, for example, in BSC-1 cells, disruption of actin network organization, loss of stress fibers and formation of F-actin-containing aggregates or foci (Schliwa, 1982). Network disruption is thought to result mainly from the filament-severing activity of the drug. Foci formation may result from secondary disorganized and uncontrolled cytoplasmic contractions (Schliwa, 1982). In neutrophils, cytochalasin D, at a concentration of $10^{-5}$ M, induced rounding up of the cells, accompanied by a coordinated reorganization of F-actin and α-actinin, and cocapping of the two proteins (Fig. 5), comparable to colocalization of α-actinin and myosin with F-actin in foci induced by cytochalasin B in PtKx cells (Wolf and Mullins, 1987).

Our data suggesting a very close association between α-actinin and F-actin in neutrophils confirm biochemical studies showing that α-actinin is retained in the Triton-insoluble cytoskeleton of neutrophils, especially after activation by the chemotactic peptide fNLPNTL (Niggli and Jenni, 1989). We also found that adhesion to glass induced a significantly increased association of α-actinin, as well as actin, with the cytoskeleton (V. Niggli, unpublished observations). In contrast to α-actinin, vinculin did not interact measurably with the Triton-insoluble cytoskeleton, even after activation of the cells with chemotactic peptide (Niggli and Jenni, 1989). A putative interaction may be too weak, and may be disrupted by the extraction conditions.

Under conditions of strong adhesion to glass, we now find evidence for an interaction of vinculin with F-actin, although different from that with α-actinin. Vinculin colocalized with F-actin in a more restricted manner than α-actinin, and, in the presence of cytochalasin D, behaved strikingly differently from α-actinin: the vinculin patches were more resistant to the drug than the α-actinin and F-actin-containing structures (Fig. 5). The possibility cannot be excluded that a small pool of actin filaments linked to vinculin may be more resistant to cytochalasin than those connected to α-actinin, which may explain this finding. Indeed, evidence has been presented for the existence of two populations of actin filaments with different sensitivity to cytochalasin B in neutrophils (Cassimeris et al., 1990). The displacement of vinculin to a cytoplasmic location by higher concentrations of cytochalasin D (Fig. 5) suggests that the organization of vinculin is also governed by that of F-actin, although not as closely as that of α-actinin. Possibly, vinculin in neutrophils interacts with actin filaments, directly or indirectly, at their barbed ends, where cytochalasin D at high concentrations can displace it. Other factors, such as interaction with specific membrane components, may also affect its organization. In contrast to our results, vinculin was shown in BSC-1 cells treated with cytochalasin D to colocalize with the resulting F-actin foci (Schliwa and Potter, 1986). Vinculin thus appears to interact differently with the actin filaments in BSC-1 cells and in adherent neutrophils. This finding may be related to a different organization of F-actin in the two cell types.

In conclusion, vinculin and α-actinin show a different location and a differential relation to the actin network in adherent human neutrophils. Vinculin may be involved in actin to membrane linkage in special adhesion sites, whereas α-actinin may be important, especially, for stabilizing pseudopods and retraction fibers by crosslinking F-actin. This study should serve as a basis for further work on the evaluation of the functional role of vinculin and α-actinin in neutrophil adhesion and migration.

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References


Cytochalasin D reveals a differential interaction of α-actinin and vinculin with the actin network

In a separate series of experiments, the relation of vinculin and α-actinin to the actin network was investigated. Double-labeling of adherent neutrophils for F-actin and α-actinin clearly revealed a close correlation of the distribution of the two proteins; they colocalized near and in retraction fibers and also in large pseudopods. The reproducible finding, that only about 50% of the F-actin-rich, non-adherent pseudopods showed an enrichment of α-actinin, suggests incorporation of α-actinin at a specific stage during dynamic reorganization of the pseudopod actin network. The effect of cytochalasin D on the location of α-actinin and F-actin confirmed their close interaction (Fig. 5). Cytochalasin D has been shown to induce, for example, in BSC-1 cells, disruption of actin network organization, loss of stress fibers and formation of F-actin-containing aggregates or foci (Schliwa, 1982). Network disruption is thought to result mainly from the filament-severing activity of the drug. Foci formation may result from secondary disorganized and uncontrolled cytoplasmic contractions (Schliwa, 1982). In neutrophils, cytochalasin D, at a concentration of $10^{-5}$ M, induced rounding up of the cells, accompanied by a coordinated reorganization of F-actin and α-actinin, and cocapping of the two proteins (Fig. 5), comparable to colocalization of α-actinin and myosin with F-actin in foci induced by cytochalasin B in PtKx cells (Wolf and Mullins, 1987).

Our data suggesting a very close association between α-actinin and F-actin in neutrophils confirm biochemical studies showing that α-actinin is retained in the Triton-insoluble cytoskeleton of neutrophils, especially after activation by the chemotactic peptide fNLPNTL (Niggli and Jenni, 1989). We also found that adhesion to glass induced a significantly increased association of α-actinin, as well as actin, with the cytoskeleton (V. Niggli, unpublished observations). In contrast to α-actinin, vinculin did not interact measurably with the Triton-insoluble cytoskeleton, even after activation of the cells with chemotactic peptide (Niggli and Jenni, 1989). A putative interaction may be too weak, and may be disrupted by the extraction conditions.

Under conditions of strong adhesion to glass, we now find evidence for an interaction of vinculin with F-actin, although different from that with α-actinin. Vinculin colocalized with F-actin in a more restricted manner than α-actinin, and, in the presence of cytochalasin D, behaved strikingly differently from α-actinin: the vinculin patches were more resistant to the drug than the α-actinin and F-actin-containing structures (Fig. 5). The possibility cannot be excluded that a small pool of actin filaments linked to vinculin may be more resistant to cytochalasin than those connected to α-actinin, which may explain this finding. Indeed, evidence has been presented for the existence of two populations of actin filaments with different sensitivity to cytochalasin B in neutrophils (Cassimeris et al., 1990). The displacement of vinculin to a cytoplasmic location by higher concentrations of cytochalasin D (Fig. 5) suggests that the organization of vinculin is also governed by that of F-actin, although not as closely as that of α-actinin. Possibly, vinculin in neutrophils interacts with actin filaments, directly or indirectly, at their barbed ends, where cytochalasin D at high concentrations can displace it. Other factors, such as interaction with specific membrane components, may also affect its organization. In contrast to our results, vinculin was shown in BSC-1 cells treated with cytochalasin D to colocalize with the resulting F-actin foci (Schliwa and Potter, 1986). Vinculin thus appears to interact differently with the actin filaments in BSC-1 cells and in adherent neutrophils. This finding may be related to a different organization of F-actin in the two cell types.

In conclusion, vinculin and α-actinin show a different location and a differential relation to the actin network in adherent human neutrophils. Vinculin may be involved in actin to membrane linkage in special adhesion sites, whereas α-actinin may be important, especially, for stabilizing pseudopods and retraction fibers by crosslinking F-actin. This study should serve as a basis for further work on the evaluation of the functional role of vinculin and α-actinin in neutrophil adhesion and migration.

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References


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