Fodrin in the human polymorphonuclear leucocyte: redistribution induced by the chemotactic peptide

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Summary

Fodrin, a membrane skeletal protein, was found to accumulate in the posterior portion of human neutrophils polarized morphologically after stimulation by the chemotactic peptide, N-formylmethionyl-leucyl-phenylalanine (FMLP). In most (>90%) unstimulated neutrophils, the distribution of fodrin was found to be uniform by immunofluorescence microscopy. When FMLP (10^{-8} M) was applied at 25°C, fodrin became polarized in about 40% of cells by 1 min, about 70% by 2 min, and about 80% by 10 min. The cells with polarized distribution decreased thereafter to about 60% of the cells at 20 min and about 20% at 60 min. Using the under-agarose system, it was confirmed that the concentration of fodrin occurred in the region opposite to the direction of chemotaxis in moving cells.

By immunoelectron microscopy, most of the labeling for fodrin was observed in the filamentous cell cortex and not associated with the plasma membrane itself. In cells polarized morphologically by FMLP, the fodrin labeling became concentrated in the posterior portion of the cell; the labeling was found most densely in the granule-rich cytoplasm, while the filamentous tail region was not labeled intensely. The lamellipodium in the head region was also labeled only sparsely.

The results indicate that in human neutrophils fodrin exists as a cytoskeletal protein rather than as a membrane protein and that the protein accumulates in the endoplasm of the posterior portion in migrating cells. The rearrangement is likely to modulate the organization of the actin-rich cell cortex for cell locomotion.

Key words: neutrophils, chemotactic peptide, fodrin, polarization, cell locomotion.

Introduction

Locomotion of polymorphonuclear leucocytes (neutrophils) is elicited by various chemotactic and chemokinetic factors. It is obvious that the cytoskeleton is engaged actively in the generation of movement, but the mechanism by which the signals received on the cell surface are transduced to the physical rearrangement of the cytoskeletal architecture is only beginning to unfold (for reviews, see Omann et al., 1987; Stossel, 1988). Since actin is undoubtedly the most important component in generating locomotion, actin-associated proteins that can affect its organization have been one focus of investigation. For example, rapid cytoskeletal association of a-actinin was reported to occur in the neutrophil after chemotactic peptide stimulation (Niggli and Jenni, 1989).

In the present study, we examined the behavior of fodrin, one of the actin-associated proteins, in relation to the neutrophil locomotion. The protein is an analogue of erythrocyte spectrin and is known to be a major calmodulin-binding protein (Kakiuchi et al., 1981). It is also a substrate of protein kinases and calpain I (for reviews, see Bennett, 1985; Marchesi, 1985; Goodman et al., 1988). Although the importance of these modifications in vivo is not fully understood, the properties suggest the possibility that fodrin may be modified in some manner upon signal reception and thereby modulate the cytoskeletal organization. In fact, translocation of fodrin from the cell membrane to other cytoplasmic sites upon receiving various stimuli has been found in lymphocytes (Black et al., 1988; Lee et al., 1988), adrenal medullary cells (Fujimoto and Ogawa, 1989b) and cultured keratinocytes (Yoneda et al., 1990a, b).

We studied the question of how the chemotactic stimulus affects distribution of fodrin in the neutrophil. The protein was found to be concentrated in the posterior end of polarized cells by immunofluorescence microscopy. Moreover, by immunoelectron microscopy fodrin was localized not in the cell membrane but in the cortical filamentous layer. These findings suggest that fodrin in the neutrophil is a cytoskeletal protein rather than a peripheral membrane protein as in most other cells. The implications of the results are discussed, especially in relation to the mechanism of cell locomotion.

Materials and methods

Antibodies

Anti-rat brain fodrin antibody was prepared and characterized as described before (Fujimoto and Ogawa, 1989a). Anti-spectrin antibody was raised in rabbits against human erythrocyte spectrin (Bennett, 1983) and purified similarly. To examine the reactivity of the antibodies, immunoblotting was carried out.
Sodium dodecyl sulfate (SDS)—polyacrylamide gel electrophoresis of solubilized neutrophils prepared using diisopropylfluorophosphate (Amrein and Stossel, 1980) and erythrocyte ghosts was run on 7.5 % gel by the method of Laemmli (1970) and proteins were electro-transferred to Dupore GVHP filter (Millipore Corp., Bedford, MA) (Towbin et al. 1979). The blots were labeled with the anti-fodrin antibody and the anti-spectrin antibody, respectively, both at 1 μg ml⁻¹ followed by peroxidase-conjugated goat anti-rabbit antibody (Cappel, West Chester, PA). Positive bands were visualized by the diaminobenzidine reaction.

**Human neutrophils**

Human neutrophils were isolated from heparinized venous blood of healthy donors by dextran sedimentation and Ficoll-Hypaque density centrifugation (Boyum, 1968). For some experiments, hypotonic shock was applied to remove residual erythrocytes. Rinses were done with 5 mM Hepes-buffered Hanks' balanced salt solution (pH 7.4) (HBSS) containing 0.1 % bovine serum albumin (BSA) (Seikagaku Kogyo, Tokyo, Japan). Cells were allowed to adhere to glass coverslips pretreated briefly with HBSS at 25°C. In some experiments, neutrophils were rinsed repeatedly with HBSS without BSA and seeded onto clean glass coverslips without pretreatment. In both cases, loosely attached cells were washed off the coverslips by pipetting HBSS mildly onto the surface.

**Stimulation with chemotactic peptide and immunofluorescence microscopy**

N-formyl-L-methionyl-leucyl-phenylalanine (FMLP; Sigma Chemical Co, St Louis, MO) was dissolved in dimethyl sulfoxide and stored frozen at −20°C in aliquots until use. The peptide was diluted to 10⁻⁸ M in HBSS containing BSA and given to neutrophils on coverslips. The final concentration of dimethyl sulfoxide was less than 0.001 % and did not affect the results at all. Control cells as well as cells incubated with the peptide for 0.5, 1, 2, 3, 5, 10, 20, 30 and 60 min at 25°C were fixed for immunofluorescence microscopy. Some cells were treated with 10 or 100 μg ml⁻¹ concanavalin A (ConA; Sigma) instead of FMLP for 10–20 min at 25°C or 37°C and fixed.

Cells were fixed with 0.5 % paraformaldehyde and 0.1 % glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 10 min at room temperature and immunolabeled using 0.5 % Triton X-100 as a permeabilizing reagent (Fujimoto and Ogawa, 1988); the primary and the secondary antibodies used were the affinity-purified anti-fodrin antibody (10 μg ml⁻¹) and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody (20 μg ml⁻¹) (Cappel), respectively. Some specimens were doubly labeled for fodrin and F-actin by adding rhodamine-phalloidin (1:80 dilution) (Molecular Probes, Junction City, OR) to the primary antibody solution. Fluorescence and phase-contrast micrographs were taken with Kodak Tri-X Pan film in an Olympus Vanox photomicroscope (Tokyo, Japan) or with a Zeiss Axiophot microscope (FRG) with epifluorescence illumination.

The percentage of cells in which fodrin accumulated in a small region was counted directly under the microscope. This seemed essential because the height of cells was variable. Cells with uniform fluorescence in one focal plane often showed polarized fodrin distribution in other planes. Cells showing clearly concentrated labeling for fodrin in any focal plane were counted as 'polarized'.

**Under-agarose system**

Agarose plates were prepared upon glass coverslips in a tissue culture dish and three round wells were cut in line as described (Nelson et al. 1976). Neutrophils were placed in the central well with 10 μl of 10⁻⁸ M FMLP in HBSS and control HBSS, both containing BSA, on opposite sides. The plates were incubated at 37°C in a humidified incubator under 5 % CO₂/95 % air for 2 h. Fixation and immunolabeling for fodrin were done in the same manner as above.

**Immunoelectron microscopy**

Neutrophils adhered to glass coverslips in the presence of BSA and incubated with FMLP for various periods of time were fixed with periodate-lysine-parafomaldehyde (PLP) solution for 3–5 h at room temperature. Erythrocytes and lymphocytes also taken from venous blood were fixed in suspension and labeled for spectrin and fodrin, respectively, in the same manner as described below. After rinses with phosphate-buffered saline (PBS) containing 10 mM glycine (PBS/glycine), they were treated with 0.1 % saponin in PBS/glycine for 30 min at 37°C, rinsed with PBS/ glycine with 0.01 % saponin (PGS), pretreated with a mixture of 0.8 % bovine serum albumin, 0.1 % gelatin and 1 % normal goat serum in PGS for 30 min at room temperature (RT). Cells were incubated with the affinity-purified anti-fodrin antibody (10–30 μg ml⁻¹) in the pretreatment protein solution for 60–90 min at 37°C. As a negative control, nonspecific rabbit IgG at the same concentration was substituted for the specific antibody. After washing, the specimens were treated with gold (1 nm)-conjugated goat anti-rabbit IgG antibody (1:40 dilution) (Janssen Biotech N.V., Olen, Belgium) in the same protein solution for 10–15 h at RT. Cells were rinsed with PBS and then with distilled water and silver enhancement was performed for 5–10 min at RT using IntenseSE M kit (Janssen) according to the manufacturer’s instructions. After rinses with distilled water, cells were dehydrated and embedded in Epoxy resin in flat molds. Coverslips were detached from the polymerized resin block by plunging into liquid nitrogen and hot water alternately, and sections were cut horizontally to the substratum. Ultrathin sections counterstained with uranyl acetate and lead citrate were observed with a JEOL 1200 EX or a JEOL 2000 EX electron microscope operated at 80 kV.

Distribution of silver-enhanced gold particles within a defined zone of a cell was quantitated according to Hartwig et al. (1989). Particles associated with the cell membrane or distributed in the most cortical 100 nm of cytoplasm were counted and their ratio to total particles per cell was calculated.

**Results**

**Immunoblotting**

By immunoblotting, the affinity-purified anti-fodrin antibody was found to recognize the α-chain of fodrin; reaction with β-chain was negligible (Fujimoto and Ogawa, 1989a). When applied to human neutrophil homogenates, the antibody reacted positively with a band of 240×10³ M₀, (Fig. 1B). The anti-spectrin antibody reacted specifically with both α- and β-chains of spectrin of the human erythrocyte ghost (Fig. 1A).

**Immunofluorescence microscopy for fodrin**

A majority of untreated neutrophils that adhered to coverslips appeared round and roughly symmetrical; fodrin was distributed evenly in most of the cells without local concentration (Fig. 2A). Only in about 6 % of the cells was a polarized distribution of fodrin observed. When FMLP was added, most neutrophils gradually became elongated; a round head and a tapered tail were unambiguously recognized. Three minutes after the FMLP addition at 25°C, cells with polarized distribution of fodrin were in the majority (Fig. 2B). The accumulation of fodrin was most apparent around 10 min after the treatment (Fig. 2D), and thereafter became less distinctive (Fig. 2E,F). The proportion of cells with local concentration of fodrin reached a plateau as early as 3 min and started to decrease after 10 min (Fig. 3). By double labeling of fodrin and F-actin, the accumulation of fodrin was shown to occur only at the opposite end to the actin-rich lamellipodia, that is, in the posterior portion of the cell (Fig. 2B,C).

Neutrophils plated on coverslips and treated with 10 or 100 μg ml⁻¹ of ConA for 10 min at either 25 or 37°C did not change shape and showed no local concentration of fodrin...
Fig. 1. Immunoblotting. Lane A, human erythrocyte ghost; and lane B, human leucocyte homogenate were run by SDS-gel electrophoresis, transferred to a filter membrane and reacted with the anti-spectrin and the anti-fodrin antibodies, respectively. The arrow indicates a band of $240 \times 10^3 M_r$.

(Fig. 2G). Cells rinsed with HBSS without BSA and placed onto coverslips in the absence of BSA remained symmetrical even after the addition of FMLP; fodrin in those cells did not accumulate locally (photograph not shown).

**Under-agarose system**

After 2 h of incubation at 37°C, cells observed under agarose between the central well and the FMLP well took on a polarized shape. Most of the cells showed accumulation of fodrin in the posterior portion facing the central well (Fig. 4A). In contrast, cells remaining in the central well were mostly unpolarized and the fodrin labeling was seen without conspicuous concentration (Fig. 4B).

**Immunoelectron microscopy**

Immunolabeling for fodrin was observed as silver-enhanced gold particles ranging from 5 to 40 nm or larger, depending upon the duration of the development. In control cells without FMLP treatment, the particles were seen scattered in the peripheral cytoplasm (Fig. 5A). Most of them were localized in the cell cortex, which appeared filamentous in nature, and were not associated with the cell membrane. Specimens incubated with nonspecific rabbit IgG for the primary antibody showed only a low background (Fig. 5B).

Human erythrocytes treated by the same protocol lost most hemoglobin, due to poor crosslinking by PLP fixative. The labeling of spectrin was found almost exclusively

**Fig. 2.** Immunofluorescence microscopy of the neutrophil labeled singly for fodrin (A,D–G) or doubly labeled for fodrin (B) and F-actin (C). A. Control; B,C, 3 min; D, 10 min; E, 20 min; F, 60 min after addition of $10^{-8}$ M FMLP at 25°C. Concentration of fodrin in the posterior region is apparent in B and D, but less obvious in E and F. In the doubly labeled cells, polarization of fodrin and F-actin in opposite directions is shown. G. 10 min after addition of $10 \mu$g ml$^{-1}$ ConA at 25°C. Fodrin is distributed without local accumulation. $\times 480$. 

**Fodrin polarization in neutrophils**
on the inner surface of the cell membrane (Fig. 5C). The particles were either adhered to the cell membrane or localized in close proximity to it. In lymphocytes, although some silver-enhanced gold particles appeared in the cytoplasm, much of the labeling was also observed either on or immediately beneath the cell membrane (Fig. 5D).

The ratio of immunolabels that are associated with the cell membrane and that are distributed within 100 nm from the membrane (including membrane-associated) was quantitated (Table 1). Whereas almost all the labeling for spectrin in erythrocytes and the majority of the labeling for fodrin in lymphocytes were localized in a narrow submembranous region, most of the labeling for fodrin in untreated neutrophils was distributed in the deeper cytoplasm.

After incubation with FMLP for 10 min, most neutrophils revealed a polarized overall morphology with a typical disposition of internal structure (Fig. 6A-C): in the head region, a filamentous lamellipodium existed, and most granules were distributed from the mid to the posterior region of the cell behind the nucleus; in the posterior portion, a filamentous cortex that was thickest in the tail was observed consistently. The particles indicating localization of fodrin were seen concentrated in the posterior portion of polarized cells. The most intense labeling was not seen in the cortical filamentous layer, but in the inner granule-rich cytoplasm. The elongated tail, mostly appearing filamentous, was not labeled intensely except for its core containing a few granules (arrows in Fig. 6). The lamellipodium consistently showed immunolabeling, but only sparsely (arrowheads in Fig. 6).

Discussion

The redistribution of fodrin observed in polarized neutrophils appears to resemble that in lymphocytes during capping that is induced by various polyvalent ligands (Levine and Willard, 1983; Nelson et al. 1983). But there are several important differences between the two phenomena. First, FMLP is a monovalent ligand, and a polyvalent lectin, ConA, which induces capping in lymphocytes, did not cause either morphological polarization or fodrin concentration in neutrophils attached to the substratum. Depolymerization of microtubules seems necessary for ConA to induce a cap in neutrophils (Albertini et al. 1977; Shereterline and Hopkins, 1981), although there has been a contradictory report (Kuehn and Epps, 1980). In any case, it is important to note that accumulation of fodrin cannot be induced by simple crosslinking of surface molecules.

Second, whereas actin as well as many actin-binding proteins coexist with fodrin beneath the lymphocyte cap, in the FMLP-treated neutrophil F-actin is distributed most densely in the lamellipodium of the head region and not in the tail (Oliver et al. 1978; Wallace et al. 1984; Haston, 1987). Myosin and actin-binding protein have also

Table 1. The ratio of immunolabeling either associated with the cell membrane or distributed in the most cortical 100 nm of the cytoplasm (including membrane-associated) in erythrocytes, lymphocytes and untreated neutrophils

<table>
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<th>Labeling adhering to the cell membrane (%)</th>
<th>Labeling either adhering to or within 100 nm from the cell membrane (%)</th>
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<tbody>
<tr>
<td>Erythrocytes</td>
<td>90.9±3.20</td>
<td>99.5±1.10</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>81.9±5.46</td>
<td>86.8±6.27</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>11.8±4.97</td>
<td>23.2±9.90</td>
</tr>
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The values are significantly different from each other (Student's t-test; P<0.001), given as mean±SD.
Fig. 5. Immunoelectron microscopy using 1 nm gold conjugate combined with silver enhancement. A. Neutrophil labeled for fodrin; without FMLP stimulation. Most of the particles are in the filamentous layer; those in the very periphery are fewer in number. ×11,000. B. Negative control. The primary antibody was substituted with nonspecific rabbit IgG. Only a few particles are seen in the cell. ×2700. C. Erythrocyte labeled for spectrin. Virtually all labels are adhered to the inner surface of the cell membrane. ×10,200. D. Lymphocyte labeled for fodrin. The majority of labels are associated with or in a close proximity of the cell membrane. ×10,400.
Fig. 6. Immunoelectron microscopy of neutrophils stimulated with FMLP for 10 min. Labeling for fodrin. The head is on the left and the tail is on the right. The labels are concentrated in the granule-rich cytoplasm of the posterior portion. The tail with thickened filamentous cortex is not intensely labeled except for its core region (arrows). The lamellipodium in the anterior portion is labeled only sparsely (arrowheads). ×6700.
been reported to be concentrated in the head of moving neutrophils (Valerius et al. 1981). In addition, morphological polarization and fodrin accumulation in neutrophils occur faster than they do in capping lymphocytes (Levine and Willard, 1983). Considering these differences as well as the cytoplasmic distribution of fodrin in the neutrophil (compared with its mostly membranous location in the lymphocyte) as discussed below, the phenomenon discussed here in the neutrophil is likely to be different from that in the capping lymphocyte.

The present method of immunoelectron microscopy using a combination of 1 nm gold-conjugated antibody and silver enhancement gave a distinct localization of the antigen. As a control experiment, we fixed and labeled human erythrocytes for spectrin by the same technique. Since spectrin in the erythrocyte is present exclusively on the cytoplasmic surface of the cell membrane, the result should provide an example of the labeling distribution expected for membrane proteins; almost all the spectrin labels were associated with the cell membrane. In contrast, only a small percentage of the immuno-labels for fodrin were seen adhered to the cell membrane of the neutrophil. Most of them were observed in the filamentous layer beneath the cell membrane, which probably corresponds to the actin-rich cell cortex described before (Bray et al. 1986; Bray and White, 1988). It is not likely that the difference in distribution seen in the erythrocyte and the neutrophil is simply due to the presence of the cytoplasm in the latter, because a majority of the fodrin labels in the lymphocyte were either adhered to the membrane or localized just beneath it. Hence, most of the fodrin molecules in the neutrophil probably exist as a cytoskeletal protein rather than as a membrane protein. In view of the similarity of locomotion of neutrophils and lymphocytes, the difference in the distribution of fodrin is surprising. The functional significance of the protein in the two cell types may be different. The results of immunoelectron microscopy also suggest that fodrin was moved to the inner cytoplasm after chemotactic peptide stimulation, although whether the protein was associated with any particular structure could not be determined.

The physiological implication of the fodrin translocation in the neutrophil can be discussed only speculatively at present. But it is most likely a phenomenon related to cell locomotion. Immobilized neutrophils on non-BSA-coated glass (Keller et al. 1979; Smith et al. 1979) did not show either morphological polarization or fodrin accumulation. The uneven distribution of fodrin may be important in establishing the functional polarization necessary for effective locomotion (Zigmond, 1989). For example, the translocation of fodrin to the endoplasm may make the filamentous cortex rigid and thus inhibit pseudopodial projections in the posterior portion; consequently protrusions occur only in the anterior portion. It is also possible that, as suggested in the exocytosis of chromaffin cells (Perrin and Aunis, 1985), the sparsity of fodrin might induce vesicles to fuse preferentially with the cell membrane in the head; vesicles carrying various receptors (Walter et al. 1980; Weinbaum et al. 1980) or recycling membrane lipids, as supposed in the membrane-flow hypothesis (Brechet, 1984; Singer and Kupfer, 1986), may be involved. The role of functional polarization has been ascribed to conventional myosin in Dictyostelium discoideum (Spudich, 1989), but myosin in the neutrophil was found concentrated in the anterior portion (Valerius et al. 1981) in contrast to the posterior localization in Dictyostelium (Yumura and Fukui, 1985). It will be interesting to study the question of whether a fodrin-like protein exists in Dictyostelium and where it is located before and after chemotactic stimulation.

It has been shown that N-formyl chemotactic receptors shift to high affinity after ligand binding and are transiently associated with the cytoskeleton (Jesaitis et al. 1984; Painter et al. 1987). Recently, the occupied receptors were shown to be segregated into actin- and fodrin-rich cell membrane domains (Jesaitis et al. 1988). The present result suggests that the receptors are concentrated in the posterior portion of the cell; the localization of fodrin in the inner cytoplasm rather than in the cell membrane or its underlying cortex implies that they may be in some intracellular compartment and not exposed on the cell surface. Accumulated fodrin may be related to endocytosis of the ligand–receptor complex (Jesaitis et al. 1984).

The molecular mechanism that causes the polarized distribution of fodrin in the neutrophil is not known. There are a few known examples in which fodrin is translocated from the cell membrane to another cytoplasmic compartment: in lymphocytes, fodrin exists as cytoplasmic aggregates (Black et al. 1988; Lee et al. 1988); in rat chromaffin cells, some retrieving vesicles during massive secretion contain fodrin (Fujimoto and Ogawa, 1989); in cultured keratinocytes, at least some of the fodrin molecules are attached to actin filaments (Yoneda et al. 1990a,b). In the above cases, however, the direct cause of fodrin translocation has not been determined. In the neutrophil stimulated with the chemotactic peptide, increase of cytoplasmic Ca²⁺ and activation of protein kinase C and calpain I are known to occur (Snyderman and Uhing, 1988; Melloni and Pontremoli, 1989) and, interestingly, all three have been shown to modify fodrin either in vitro, in other cells or in both (Marchesi, 1985; Goodman et al. 1988; Melloni and Pontremoli, 1989). Molecular details of how fodrin is chemically changed or not changed in moving cells and how the translocation is caused obviously merit further study, both for delineation of the role of fodrin and for understanding of the cell locomotory mechanism.

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References


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