Association of Ligand-Receptor Complexes with Actin Filaments in Human Neutrophils: A Possible Regulatory Role for a G-Protein

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Abstract. Most ligand-receptor interactions result in an immediate generation of various second messengers and a subsequent association of the ligand-receptor complex to the cytoskeleton. Depending on the receptor involved, this linkage to the cytoskeleton has been suggested to play a role in the termination of second messenger generation and/or the endocytic process whereby the ligand-receptor complex is internalized. We have studied how the binding of chemotactic peptide-receptor complexes to the cytoskeleton of human neutrophils is accomplished. As much as 76% of the tritiated formylmethionyl-leucyl-phenylalanine (fMet-Leu-[^3H]Phe) specifically bound to intact cells, obtained by a 30-s stimulation with 20 nM fMet-Leu-[^3H]Phe, still remained after Triton X-100 extraction. Preincubating intact cells with dihydrocytochalasin B (dhCB) or washing the cytoskeletal preparation with a high concentration of potassium, reduced the binding of ligand-receptor complexes to the cytoskeleton by 46% or more. Inhibition of fMet-Leu-Phe-induced generation of second messengers by ADP-ribosylating the α-subunit of the receptor-coupled G-protein with pertussis toxin, did not reduce the binding of ligand-receptor complexes to the cytoskeleton. However, using guanosine-5′-O-(2-thiodiphosphate) (GDPβS) to prevent the dissociation of the fMet-Leu-Phe-associated G-protein within electrically permeabilized cells, led to a pronounced reduction (62%) of the binding between ligand-receptor complexes and the cytoskeleton. In summary, in human neutrophils the rapid association between chemotactic peptide-receptor complexes and the cytoskeleton is dependent on filamentous actin. This association is most likely regulated by the activation and dissociation of the fMet-Leu-Phe-associated G-protein.

Human neutrophils and macrophages play an important role in the body defense against microorganisms. A fundamental quality of these cells, enabling them to perform these duties, is their ability to move and engulf particles. These motile events are dependent on dynamic alterations and reorganization of their cytoskeleton (36). The motile activity is induced by the specific binding of a ligand, a chemotactic factor or a phagocytic opsonin, to its respective receptor. The formation of ligand-receptor complexes triggers the generation of various transmembrane signals responsible for the activation of different effector systems in these cells (35). The signal transduction system activated by chemotactic factors in human neutrophils is generally agreed to involve a guanine nucleotide binding protein, G-protein (15). Evidence for the involvement of this G-protein is usually tested by using pertussis toxin (1, 4, 8, 25, 31), which ADP-ribosylates and thereby inhibits the activity of the α-subunit of the G-protein (9, 24). In the absence of pertussis toxin the chemotactic factor-receptor complex, via the α-subunit of the G-protein, causes an increase in the activity of phospholipase C. This results in an increased breakdown of phosphatidylinositol 4,5-bisphosphate (PtdIns (4,5)P₂) and a subsequent accumulation of inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) and diacylglycerol (18, 37). It is generally agreed, in studies of a number of different cell types, that Ins(1,4,5)P₃ is responsible for the mobilization of Ca²⁺ from intracellular stores, whereas diacylglycerol is the natural activator of protein kinase C (7, 30).

The ability of the chemotactic peptide-receptor complex to generate transmembrane signals responsible for the subsequent cell responses has been hypothesized to be modulated by the cytoskeleton (21, 22). A few seconds after a chemotactic factor-receptor complex is formed it becomes associated with the cytoskeleton (20). It has been suggested that this cytoskeletal interaction with the ligand-occupied receptor serves as a negative-feedback mechanism, terminating the formation of transmembrane signals (21, 22). In addition, it could also have an equally important function by representing the first step in the endocytic process whereby the ligand-receptor complex is internalized. Although it has been suggested that the cytoskeleton plays a role in receptor function, the mechanism(s) behind the association between chemotactic receptors and the cytoskeleton is presently unknown (34).

Consequently, the aim of the present investigation was to
elucidate further how the association between the fMet-Leu-
Phe–receptor complex and the cytoskeleton is achieved and
to examine the possible role of the fMet-Leu-Phe–activated
signal transduction system in this process.

Materials and Methods

Chemicals

All reagents used were of an analytical grade. Dextran and Ficoll-Paque
were from Pharmacia Fine Chemicals (Uppsala, Sweden). Dihydrocyto-
chalasin B (dCB) and fMet-Leu-Phe were both obtained from Sigma
Chemical Co. (St. Louis, MO). GDP/βS was purchased from Boehringer
Mannheim (Mannheim, FRG). Fluorescein-phalloidin was from Molecular
Probes Inc. (Eugene, OR). Pertussis toxin was from List Biological Labora-
tories Inc. (Campbell, CA). New England Nuclear (Boston, MA) provided
fMet-Leu-[3H]Phe and [35S]GTP. The FITC-conjugated goat anti–mouse an-
tiser was obtained from Cappell Laboratories (Malvern, PA). The
monoclonal tubulin-antibodies were a generous gift from Professor Peter
Collins (the Ludwig Institution, KI, Stockholm, Sweden).

Isolation of Neutrophils

Blood was obtained from healthy volunteers and collected in heparin-con-
taining vacutainer tubes. After sedimentation on dextran the neutrophils
were isolated according to the method described by Böyum (11). Contamin-
ating erythrocytes were removed by a 20–30-s hypotonic lysis in distilled
water, after which the cell suspension was centrifuged on a Ficoll-Paque
gradient to separate the polymorphonuclear leucocytes from lymphocytes,
monocytes and platelets. The neutrophils were then washed twice before
resuspension in a calcium-containing medium with the following compo-
sition: 136 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.1 mM CaCl2, 0.1
mM MgATP, 1.2 mM KH2PO4, 50 mM NaHCO3, 5.5 mM glucose and 20
mM Hepes, pH 7.4.

Neutrophil Permeabilization

The cells were suspended at a concentration of 107/ml in a medium with
the following composition: 20 mM NaCl, 100 mM KCl, 1 mM Na2HPO4,
25 mM NaHCO3, 20 mM Hepes, 1 mM EGTA, and 0.2% (wt/vol) BSA,
pH 7.0. This medium will be referred to as "permeabilization medium." The
cells were rendered permeable by 25–30 exposures (150 s each) to an elec-
tric field of 1.7 kV/cm. This was carried out in equipment designed by
Professor E. Gylfe, of the Department of Cell Biology, University of Upp-
sala, Sweden, and manufactured by the Medical Technical Centre at
the University of Linköping, Sweden. During the permeabilization procedure
the neutrophils were kept on ice and were stirred gently between every five
pulses using a plastic pipette; after this treatment, the cells were stored on
ice. The degree of permeabilization was estimated by using 86Rb
do, trypan blue staining. Trypan blue dissolved in 0.9% NaCl (12.5 µg/ml)
was added to the permeabilized cells at a ratio of 1:1; the cells were then allowed to stand for 10 min at +4°C.
<4% of control cells, which had been treated with trypan blue in the same
manner but had not been permeabilized, were stained. The amount of cyto-
solic LDH remaining in the permeabilized cells was determined by mea-
suring the consumption of NADH in a spectrophotometer at 340 nm for
6 min (6).

Receptor-binding Assay

Neutrophils (5.5 × 106/ml) were suspended in 0.45 ml of the calcium-
containing medium and placed in microcentrifuge tubes. The tubes were
1. Abbreviations used in this paper: dCB, dihydrocytochalasin B; F-actin,
filamentous actin; fMet-Leu-Phe, N-formyl-L-methionyl-L-leucyl-L-phenyl-
amine; fMet-Leu-[3H]Phe, tritiated N-formyl-L-methionyl-L-leucyl-L-phe-
nylalanine; GDP/βS, guanosine-5'-O-(2-thiodiphosphate); Ins(1,4,5)P3, inos-
stol 1,4,5-trisphosphate; LDH, lactate dehydrogenase; PtdIns(4,5)P2, phos-
phatidylinositol 4,5-bisphosphate.

transferred to a water bath (37°C) for a 5-min equilibration period. There-
after, the cells were stimulated with 20 nM fMet-Leu-[3H]Phe in the
presence or absence of 20 µM unlabeled fMet-Leu-Phe. The binding was stopped
after 30 s by putting the cells on ice and simultaneously adding ice-cold
medium. Nonspecific binding was defined as the amount of binding that oc-
curred in the presence of 20 nM unlabeled fMet-Leu-Phe. Specific binding
was defined as total binding minus nonspecific binding. The value obtained
from one single experiment is based on 2–3 determinations per batch of cells.

Preparation of the Cytoskeleton

An ice-cold medium containing Triton X-100 (final concentration 1.0%;
vol/vol) was added to the cells. This procedure has previously been used
(39), since it essentially leaves only the cytoskeleton intact. Triton X-100
was always dissolved in a medium with the following composition: 100 mM
KCl, 10 mM EGTA and 20 mM trizma-base, pH 7.0. The samples were rap-
idly mixed and put on ice for 10 min, after which the suspension was cen-
trifuged for 20 s at ~9000 g in a microfuge (Beckman Instruments, Inc.,
Palo Alto, CA). The pellets were then resuspended in the medium described
above, but without Triton X-100, and kept on ice for 10 min, before an addi-
tional centrifugation was performed. To enable determination of fMet-Leu-
[3H]Phe binding to cells treated in this manner, the final pellets were
resuspended in 0.5 ml soluen for 18 h at room temperature, and then trans-
ferred to scintillation vials, containing 2.5 ml distilled water and 5 ml
aqnasol. The radioactivity was determined in a liquid scintillation counter.

Morphological Examinations

Neutrophils suspended in the calcium-containing medium (10/ml), not ex-
posed to fMet-Leu-Phe, were plated onto Formvar-coated gold grids or stan-
dard cover slips. The cells were allowed to settle for 30 min at 37°C. The
medium and nonadherent cells were removed, and the attached cells were
incubated in an ice-cold medium containing Triton X-100 for 10 min at
+4°C. Adherent cells were chosen for the morphological examinations,
since they can be used for testing the effects of a high potassium concentra-
tion on the cellular content of F-actin and tubulin. Control experiments
showed that cells in suspension did not differ significantly in appearance
from those presented in this study. The attached cytoskeleton preparations
were washed once for 10 min (+4°C). The preparations for fluorescent
identification of F-actin and tubulin were fixed in paraformaldehyde (4.0% w/t/vol; pH 7.3), whereas the samples for EM were fixed in glutaraldehyde
(2.5% w/vol) in 0.1 M sucrose and 0.1 M sodiumacetoclyde, pH 7.2. These
fixation procedures were carried out on ice for 15 min and then for an addi-
tional 30 min at room temperature. The samples were then stored in the fixa-
tive at +4°C until further processing.

Actin Staining. The cytoskeletal preparations were rinsed in PBS (2 ×
5 min). The cover slips were then placed in a moist chamber, and incu-
bated in a solution of Fluorescein-phalloidin (0.6 µg/ml) in the dark for
30 min at room temperature (4°C). The stained preparations were then washed
twice in PBS (2 × 5 min) and once in distilled water (5 min), after which
they were mounted on glass slides with gelvatol. A microscope (Carl Zeiss,
Inc., Oberkochen, FRG) equipped for fluorescence (objective 40×) was used
for examination and photography (film: Tri-X Pan 400 developed at
800 ASA; Eastman Kodak Co., Rochester, NY).

Tubulin Staining. The cytoskeletal preparations were rinsed in PBS (2 ×
5 min) and then in TBS-BSA (TBS containing 1% BSA; 2 × 5 min). The
cover slips were then placed in a moist chamber, and incubated with mono-
clonal antibasinulin antibodies (2) for 45 min at 37°C. The preparations were then washed twice in TBS-BSA (2 × 5 min) before they were placed in
the moist chamber for an additional incubation with a FITC-conjugated goat
–mouse antisera for 45 min at 37°C. After incubation the preparations
were washed twice in TBS-BSA (2 × 5 min) and once in PBS (5 min), be-
fore they were mounted as described above. Nonspecific fluorescence could
be excluded by incubating samples with mouse antisera and FITC-con-
jugated goat anti–mouse antisera. A microscope (Carl Zeiss, Inc.) equipped
for fluorescence (objective 63×) was used for examination and photography
(film: Tri-X Pan 400 developed at 800 ASA; Eastman Kodak Co., Roches-
ter, NY).

Electron Microscopy. After fixation, the grids with the cytoskeletal
preparations were prepared for EM as previously described (3, 27). In brief,
the grids were rinsed with a 0.15 M sodiumacetoclyde buffer and then dipped
several times in each of the three different volumes of distilled water. The sam-
plees were frozen in propane, cooled to liquid nitrogen temperature and
freeze dried over night at ~70°C. The next day the specimen chamber was
warmed up to +30°C and taken to atmospheric pressure by bleeding in dry
argan gas. The grids were coated with 1.5 nm tungsten (W) in a magnetron sputter coater (29) installed in a vacuum evaporator equipped with a quartz crystal thickness monitor. Photographs were taken in an electron microscope (2000 Ex TEMSCAN; JEOL, Tokyo, Japan) operated at 160 kV in scanning mode.

Results
Morphological Examination of the Cytoskeletal Preparation

Intact neutrophils (Figs. 1, A–C) or neutrophils rendered permeable (Fig. 1 D) were allowed to settle on standard cover slips at 37°C for 30 min. The adherent cells were then treated with ice-cold Triton X-100. The cells shown in Fig. 1 A exhibited strong fluorescence, indicating that the Triton extraction had no major disrupting effect on F-actin. Cells in Fig. 1 B, which were Triton treated and also exposed to a high concentration of potassium (600 mM), exhibited almost no fluorescence. Cells pretreated with dhCB (40 μM; Fig. 1 C) before preparation of the cytoskeleton were also well stained for F-actin, although the actin network exhibited a somewhat reduced concentration of potassium (600 mM), which is exhibited almost no permeability (Fig. 1 D) were allowed to settle on standard cover slips at 37°C for 2 h at 37°C. After incubation the cells were resuspended (5.5 × 10⁶/ml) and stimulated with 20 μM fMet-Leu-Phe for 30 s at 37°C. The reactions were immediately stopped by addition of ice-cold paraformaldehyde (3.2%; w/vol). The mixtures were stored at 4°C until staining with fluorescein-phalloidin. The samples were stained with a 100 μl mixture of fluorescein-phalloidin (0.5 μg/ml) and lysophosphatidylcholine (0.1 mg/ml) for 30 min in the dark at room temperature. The stained samples were then washed with PBS after which 350 μl absolute methanol was added. Methanol-extraction was carried out for 1 h in the dark, with frequent interruptions for vortexing. The extracts were centrifuged, after which the fluorescence of the supernatant was determined in a spectrofluorometer (LS-3B; Perkin Elmer Corp., Beaconsfield, UK; with excitation set at 488 nm and emission at 522 nm).

F-actin Determination

The cellular content of F-actin was analyzed by staining with fluorescein-phalloidin, essentially as previously described (4, 5, 19, 38). In short, neutrophils were suspended in the calcium-containing medium (10⁷/ml) and incubated with or without pertussis toxin (450 ng/ml) for 2 h at 37°C. After incubation the cells were resuspended (5.5 × 10⁶/ml) and stimulated with 20 μM fMet-Leu-Phe for 30 s at 37°C. The reactions were immediately stopped by addition of ice-cold paraformaldehyde (3.2%; w/vol). The mixtures were stored at 4°C until staining with fluorescein-phalloidin. The solutions were stained with a 100 μl mixture of fluorescein-phalloidin (0.5 μg/ml) and lysophosphatidylcholine (0.1 mg/ml) for 30 min in the dark at room temperature. The stained samples were then washed with PBS after which 350 μl absolute methanol was added. Methanol-extraction was carried out for 1 h in the dark, with frequent interruptions for vortexing. The extracts were centrifuged, after which the fluorescence of the supernatant was determined in a spectrofluorometer (LS-3B; Perkin Elmer Corp., Beaconsfield, UK; with excitation set at 488 nm and emission at 522 nm).

Effects of Potassium and dhCB on the Specific Binding of fMet-Leu-[3H]Phe to the Cytoskeleton

Neutrophils were stimulated with fMet-Leu-[3H]Phe for 30 s and then treated with ice-cold Triton X-100. In comparison with intact cells, the specific binding of fMet-Leu-Phe was reduced by ~25% in the cytoskeletal preparations obtained after Triton X-100 treatment. In one series of experiments, the obtained cytoskeletal preparations were exposed to a high concentration of potassium (600 mM; Table I), which is known to predominantly disrupt filamentous actin (12, 26). This potassium-induced disruption of actin filaments led to a substantial loss of specifically bound fMet-Leu-Phe. To further explore the role of actin in the association of ligand-receptor complexes to the cytoskeleton, cells were pretreated with dhCB (40 or 60 μM) prior to stimulation with fMet-Leu-[3H]Phe and exposure to ice-cold Triton X-100. As shown in Table I, the number of specifically bound ligands (associated with the cytoskeletal preparation) was reduced by ~60%, regardless of the dhCB concentration.

Effects of Pertussis Toxin on the Association of Specifically Bound fMet-Leu-[3H]Phe-receptor Complexes with the Cytoskeleton

Neutrophils preincubated with different concentrations of pertussis toxin were stimulated with fMet-Leu-[3H]Phe and then immediately exposed to ice-cold Triton X-100. As shown in Fig. 4, no significant reduction in the binding of ligand-receptor complexes to the cytoskeletal preparation could be observed with any of the concentrations tested. This batch of pertussis toxin (450 ng/ml) totally inhibited the fMet-Leu-Phe–induced polymerization of actin in these cells (Fig. 4, inset). In addition it also abolished the fMet-Leu-Phe–induced rise in cytotoxic free calcium and the generation of InsP₃, as well as the production of O₂⁻-ions, even at a concentration as low as 200 ng/ml (data not shown), as previously described (25).

Electro-permeabilization of Neutrophils

Neutrophils were permeabilized, to enable introduction of GDP/βS, by repeated electric discharges (1.7 kV/cm; 150 ms each) at +4°C. After permeabilization, the efflux of ³⁸Rb⁺ (molecular weight of 86) and LDH (molecular weight of 140,000) and the uptake of trypan blue (molecular weight of 961) were used as markers to evaluate the degree of permeabilization. Only a negligible decrease in the content of the cytotoxic enzyme LDH can be noticed, even after a large number of discharges, whereas the efflux of ³⁸Rb⁺ was almost complete after only a few discharges (Fig. 5). The number of cells stained with trypan blue steadily increased with the number of electric discharges. Batches with 30–75% trypan blue staining were found to be best suited for the subsequent experimental work; batches of cells that, after permeabilization, showed a trypan blue staining outside of this range were consequently discarded. Based on these findings, we normally permeabilized the cells with 25–30 electrical discharges. On average, the electro-permeabilization alone reduced the specific binding of fMet-Leu-Phe by 28 ± 7%.

Effect of GDP/βS and On the Specific Binding of fMet-Leu-[3H]Phe-receptor Complexes to the Cytoskeleton

Electro-permeabilized neutrophils, incubated with or with-
Figure 1. Photographs of cytoskeletal preparations stained with fluorescein-phalloidin. The cells were allowed to attach to standard cover slips and were then washed with Triton X-100. The obtained preparations were fixed in paraformaldehyde, after which they were stained with fluorescein-phalloidin (0.6 μg/ml). A shows a cytoskeletal preparation; i.e., cells treated only with Triton X-100. Cytoskeletal preparations washed with a high concentration of potassium (600 mM) are shown in B, and cells incubated with dbCB (40 μM) and subsequently washed with Triton X-100 are shown in C. Finally, permeabilized cells incubated with GDP/βS (1 mM) and subsequently washed with Triton X-100 are shown in D.
Figure 2. Photographs of cytoskeletal preparations labeled with monoclonal antitubulin antibodies and FITC-conjugated goat anti-mouse antisera. The cells were allowed to attach to standard cover slips and were then washed with Triton X-100. The obtained preparations were fixed in paraformaldehyde, after which they were labeled as described in the Materials and Method section. A shows a cytoskeletal preparation; i.e., cells treated only with Triton X-100. Cytoskeletal preparations washed with a high concentration of potassium (600 mM) are shown in B, and cells preincubated with dHB (40 μM) and subsequently washed with Triton X-100 are shown in C.
Figure 3. Electron micrographs of human neutrophils treated with ice-cold Triton X-100. The cells were allowed to attach to Formvar-coated gold grids before the ice-cold Triton-100 treatment was performed. The cytoskeletal preparations were fixed in glutaraldehyde and thereafter prepared for scanning EM. A single representative cell is shown at two different magnifications: (A) 15,000 and (B) 40,000.
Preparations and resuspension of the cytoskeletal preparations, obtained from cells stimulated with dhCB at concentrations of 40 and 60 μM, respectively.

Permeabilized cells were put on ice and exposed to GDP/3S potassium (100 mM; controls). The effect of dhCB was tested by preincubating intact cells with dhCB (40 or 60 μM) at 37°C for 10 min followed by stimulation with 20 nM fMet-Leu-[3H]Phe. The number of specifically bound ligand-receptor complexes associated with the cytoskeleton, in the cytoskeletal preparations treated with 600 mM potassium, is expressed as percentage of the value obtained from cells treated with Triton X-100 and a normal concentration of potassium (100 mM; controls). The effect of dhCB was tested by preincubating intact cells with dhCB (40 or 60 μM) at 37°C for 10 min followed by stimulation with 20 nM fMet-Leu-[3H]Phe. The number of specifically bound ligand-receptor complexes associated with the cytoskeleton preparation is expressed as percentage of the value obtained in parallel from cells not exposed to dhCB before the Triton X-100 extraction (controls). The effect of GDP/3S (1 mM) on the specific binding of fMet-Leu-Phe to the cytoskeleton was tested in electroporation-permeabilized cells. Permeabilized cells were put on ice and exposed to GDP/3S for 10 min and then incubated for an additional 10 min in a waterbath (37°C) before stimulation with 20 nM fMet-Leu-[3H]Phe. The number of specifically bound ligand-receptor complexes associated with the cytoskeleton is expressed as a percentage of the value obtained from similarly permeabilized cells not exposed to GDP/3S (controls). The radioactivities in the control groups of the potassium, dhCB, and GDP/3S experiments were calculated to (3.4 ± 0.3) x 10^3 dpm, (1.7 ± 0.3) x 10^3 dpm and (1.8 ± 0.2) x 10^3 dpm, respectively. Values in this table are mean ± SEM for n = 5–7.

Table I. Specific Binding of [3H]MLP to Cytoskeletal Preparations

| Incubation conditions | Percentage of control |
|-----------------------|-----------------------|
| KCl 600 mM            | 53.7 ± 7.5            |
| dhCB 40 μM            | 36.0 ± 4.6            |
| dhCB 60 μM            | 42.6 ± 6.0            |
| GDP/3S 1 mM           | 38.0 ± 6.1            |

Effects of potassium, dhCB, and GDP/3S on the specific binding of fMet-Leu-Phe to the cytoskeleton. The effect of potassium was tested by centrifugation and resuspension of the cytoskeletal preparations, obtained from cells stimulated with 20 nM fMet-Leu-[3H]Phe, in a Triton-lacking medium with a potassium concentration of 600 mM. The number of specifically bound ligand-receptor complexes associated with the cytoskeleton, in the cytoskeletal preparations treated with 600 mM potassium, is expressed as percentage of the value obtained from cells treated with Triton X-100 and a normal concentration of potassium (100 mM; controls). The effect of dhCB was tested by preincubating intact cells with dhCB (40 or 60 μM) at 37°C for 10 min followed by stimulation with 20 nM fMet-Leu-[3H]Phe. The number of specifically bound ligand-receptor complexes associated with the cytoskeleton preparation is expressed as percentage of the value obtained in parallel from cells not exposed to dhCB before the Triton X-100 extraction (controls). The effect of GDP/3S (1 mM) on the specific binding of fMet-Leu-Phe to the cytoskeleton was tested in electroporation-permeabilized cells. Permeabilized cells were put on ice and exposed to GDP/3S for 10 min and then incubated for an additional 10 min in a waterbath (37°C) before stimulation with 20 nM fMet-Leu-[3H]Phe. The number of specifically bound ligand-receptor complexes associated with the cytoskeleton is expressed as a percentage of the value obtained from similarly permeabilized cells not exposed to GDP/3S (controls). The radioactivities in the control groups of the potassium, dhCB, and GDP/3S experiments were calculated to (3.4 ± 0.3) x 10^3 dpm, (1.7 ± 0.3) x 10^3 dpm and (1.8 ± 0.2) x 10^3 dpm, respectively. Values in this table are mean ± SEM for n = 5–7.

Figure 4. Effect of pertussis toxin on the subsequent binding between ligand-receptor complexes and the cytoskeletal preparation. Neutrophils were suspended in calcium-containing medium (107/ml) and incubated for 2 h at 37°C with different concentrations of pertussis toxin (indicated in the figure). The cells were then washed, resuspended (5.5 x 10^6 cells/ml) and preincubated for 5 min at 37°C. This was followed by stimulation with 20 nM fMet-Leu-[3H]Phe for 30 s (with or without an excess of unlabeled ligand) and treatment with Triton X-100, as previously described. The number of specifically bound ligand-receptor complexes associated with the cytoskeleton is expressed as a percentage of the value obtained in parallel from cells not exposed to any additives before the Triton X-100 extraction (control). The activity in the control group was (3.1 ± 0.2) x 10^3 dpm. Values are mean ± SEM for n = 4–7. The inset shows the effect of pertussis toxin (450 ng/ml for 2 h at 37°C) on the fMet-Leu-Phe−induced polymerization of actin. The cells were resuspended (5.5 x 10^6/ml) and stimulated with 20 nM fMet-Leu-Phe for 30 s. The stimulation was stopped by addition of an ice-cold paraformaldehyde solution. The cellular content of F-actin was then determined with fluorescein-phalloidin. Values are mean ± SEM for n = 5.

Figure 5. Electro-permeabilization of intact neutrophils. Cells were suspended in a permeabilization medium (10^7/ml) and rendered permeable by an increasing number of exposures (150 ms each) to an electric field of 1.7 kV/cm. After permeabilization, the remaining contents of LDH (○) and 48Rb+ (□) and the number of trypan blue−stained cells (△) were determined. The amount of LDH and 48Rb+ left in the cells after permeabilization is expressed as a percentage of the value obtained from nonpermeabilized cells (control cells). Each separate LDH value is the mean of two different determinations from the same batch of cells. The amount of trypan blue−stained neutrophils (expressed as a percent) were obtained after counting 100−200 permeabilized cells. Values are mean ± SEM for n = 3−5.

Discussion
The purpose of this study was to examine how the association between the fMet-Leu-Phe-receptor complex and the cytoskeleton in human neutrophils is accomplished and to examine the possible role of the fMet-Leu-Phe-activated signal transduction system in this process.

In the present study a high concentration of potassium was shown to reduce the specific binding of fMet-Leu-Phe to the cytoskeletal preparation by ∼50%. In addition, previous investigations by other authors (12, 26) and results of this present study demonstrate that the same concentration of potassium mainly disrupts filamentous actin, and does so quite effectively. We have demonstrated that a high potassium concentration leads to a pronounced reduction of F-actin staining in cytoskeletal preparations, whereas it has only a minor effect on the staining of tubulin in these preparations. This suggests that filamentous actin plays a role in the binding process between fMet-Leu-Phe-receptor complexes and the cytoskeleton. The fungal metabolite cytochalasin B is generally considered to bind preferentially to the free barbed ends of actin, and does so quite effectively. We have demonstrated that a high potassium concentration leads to a pronounced reduction of F-actin staining in cytoskeletal preparations, whereas it has only a minor effect on the staining of tubulin in these preparations. This suggests that filamentous actin plays a role in the binding process between fMet-Leu-Phe-receptor complexes and the cytoskeleton. The fungal metabolite cytochalasin B is generally considered to bind preferentially to the free barbed ends of F-actin molecules (13, 16). Because of this property, cytochalasin B inhibits fMet-Leu-Phe−induced polymerization of actin in human neutrophils (38). Consequently, the reduced binding between ligand-receptor complexes and the cytoskeleton in the presence of cytochalasin B, also suggests a role for actin in this process. The mechanism behind this effect of cytochalasin B could either be an abolished polymerization of actin or the absence of available binding sites.
on the F-actin network. However, it could be demonstrated that pertussis toxin, another inhibitor of f-Met-Leu-Phe-induced polymerization of actin in neutrophils (4, 33, and data in this paper), did not reduce the binding of f-Met-Leu-Phe-receptor complexes to the cytoskeletal preparation. Using a different technical approach and a slightly different ligand, Painter et al. (32) found that dhCB but not pertussis toxin affected the cellular processing of ligand-receptor complexes. Pertussis toxin inhibits the f-Met-Leu-Phe transduction system by ribosylation of the G-protein involved. The most likely explanation for pertussis toxin–induced inhibition of actin polymerization is not a direct effect of the toxin on the cytoskeleton but instead an impaired generation of vital second messengers. Consequently, previous and present findings suggest that the association between f-Met-Leu-Phe–receptor complexes and the cytoskeleton are dependent on the availability of binding sites on the actin network rather than on polymerization per se.

Total abolishment of binding of ligand-receptor complexes to the cytoskeletal preparation was not obtained in any of the experimental situations discussed above. This observation could be explained by at least three different mechanisms. (a) It has been demonstrated that some F-actin molecules still remain after exposure to a high concentration of potassium (12), and that cytochalasin B does not totally block all available free ends on F-actin (10). (b) In the cytoskeletal preparations used, small membrane residues may still remain; it is possible that these residues might possess ligand-receptor complexes not associated with the cytoskeleton and, consequently, not affected by manipulations of the cytoskeleton. (c) It is also possible that some other cytoskeletal component(s), in addition to filamentous actin, participates in the processing of the chemotactic peptide-receptor complexes. Regardless of which mechanism that interacts with our method, it does not affect the conclusions made from the data obtained in the present study.

In human neutrophils, the generation of transmembrane signals is believed to occur before chemotactic factor–receptor complexes become associated with the cytoskeleton (21). Consequently, it seems logical to suggest that one or more of these signals is responsible for the subsequent binding between ligand-receptor complexes and the cytoskeleton. Pertussis toxin is known to totally inhibit f-Met-Leu-Phe–induced generation of transmembrane signals in human neutrophils via ribosylation and inactivation of the G-protein linking the f-Met-Leu-Phe–receptor complex to phospholipase C (25, 31). For this reason pertussis toxin seems ideal for testing a possible role of any of these transmembrane signals in the binding process between ligand-receptor complexes and the cytoskeleton. Pertussis toxin is known to totally inhibit f-Met-Leu-Phe–induced binding of transmembrane signals in human neutrophils (23). However, it was recently demonstrated that pertussis toxin did not prevent the dissociation between the β- and the γ-subunits, even if the α-subunit was efficiently ribosylated (28). Consequently, any involvement of the dissociation process itself or the γ-subunit in cellular regulation can not be adequately tested with pertussis toxin. To circumvent this problem in the present study we used GDPβS, a stable GDP analogue that competitively inhibits GTP-induced dissociation and activation of G-proteins (15, 17). When permeabilized cells were treated with GDPβS, a pronounced reduction of the association between chemotactic factor-receptor complexes and the cytoskeleton was observed. The possibility of a nonspecific effect of GDPβS on the amount of F-actin is negligible, since no reduction in fluorescein-phalloidin staining could be detected in permeabilized cells exposed to GDPβS. Our data suggest that either the βγ-subunit directly or the dissociation and release of either the α-subunit or the βγ-subunit triggers the binding between the ligand-receptor complex and the cytoskeleton. Considering the first possibility, results of the present study do not indicate whether the βγ-subunit is part of the ligand-receptor–cytoskeleton complex or if this subunit only regulates the formation of this complex. Recent results of other investigators suggest that such complexes do not interact directly with a G-protein (23). However, a direct association between the β-unit of a G-protein and the cytoskeleton has been demonstrated in S49 mouse lymphoma cells (14). The alternative explanation, in which the dissociation of the G-protein is the crucial event, could be that the dissociation and release of either the α-subunit or the βγ-subunit unmasks a certain binding site on the receptor thereby causing a conformational change in the receptor. This could trigger the binding of a ligand-receptor complex to the cytoskeleton. Obviously, additional experiments are required to determine the precise mechanism by which the G-protein participates in the binding between the f-Met-Leu-Phe–receptor unit and the microfilamentous system in human neutrophils.

In summary, the present data demonstrate that the f-Met-Leu-Phe–receptor complex rapidly associates with the F-actin component of the cytoskeleton, even in the absence of actin polymerization, and that the G-protein involved in the f-Met-Leu-Phe transduction system most likely participates in the regulation of this binding process.

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References

1. Andersson, T., W. Schlegel, A. Monod, K.-H. Krause, O. Stendahl, and D. P. Lew. 1986. Leukotriene B4 stimulation of phagocytes results in the formation of inositol 1,4,5-trisphosphate. Biochem. J. 240:333-340.
2. Bell, P. B., I. Rundquist, I. Svensson, and V. P. Collins. 1987. Use of cytofluorometry to evaluate binding of antibodies to the cytoskeleton of cultured cells. J. Histochem. Cytochem. 35:1381-1388.
3. Bell, P. B., M. Lindroth, and B.-A. Fredriksson. 1988. Preparation of cytoskeletons of cells in culture for high resolution scanning and scanning transmission electron microscopy. Scanning Microsc. 2:1647-1661.
4. Bengtsson, T., O. Stendahl, and T. Andersson. 1986. The role of the cytosolic free Ca2+ transient for f-Met-Leu-Phe-induced actin polymerization in human neutrophils. Eur. J. Cell Biol. 42:338-343.
5. Bengtsson, T., I. Rundquist, O. Stendahl, M. P. Wymann, and T. Andersson. 1988. Increased breakdown of phosphatidylinositol 4,5-bisphosphate is not an initiating factor for actin assembly in human neutrophils. J. Biol. Chem. 263:17383-17389.
6. Bergmeyer, H. U., and E. Bernt. 1963. Lactate dehydrogenase. In Methods in Enzymatic Analysis. H. U. Bergmeyer, editor. Academic Press Inc., New York. 731-739.
7. Berdrige, M. J. 1987. Inositol trisphosphate and diacylglycerol: two interacting second messengers. Annu. Rev. Biochem. 56:159-193.
8. Bokoch, G. M., T. Kataoka, J. K. Northup, E. L. Hewlett, and A. G. Gilman. 1983. Identification of the predominant substrate for ADP-ribosylation by islet activating protein. J. Biol. Chem. 258:2072-2075.
10. Bonder, E. M., and M. S. Mooseker. 1986. Cytochalasin B slows but does not prevent monomer addition at the barbed end of the actin filament. J. Cell Biol. 102:282–288.

11. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. 97:77–89.

12. Bravo, R., J. V. Small, S. J. Fey, P. M. Larsen, and J. E. Celis. 1982. Architecture and polypeptide composition of HeLa cytoskeletons, modification of cytoarchitectural polypeptides during mitosis. J. Mol. Biol. 154:121–143.

13. Brown, S. S., and J. A. Spudich. 1981. Mechanism of action of cytochalasin: evidence that it binds to actin filament ends. J. Cell Biol. 88:487–491.

14. Carlson, K. E., M. J. Woolkalis, M. G. Newhouse, and D. R. Manning. 1987. Polyphosphoinositide phospho-diesterase: regulation by a novel guanine nucleotide binding protein. J. Biol. Chem. 262:15900–15905.

15. Cockcroft, S. 1987. Polyphosphoinositide phospho-diesterase: regulation by a novel guanine nucleotide binding protein. G Protein. Trends Biochem. Sci. 12:75–78.

16. Cooper, J. A. 1987. Effects of cytochalasin and phalloidin on actin. J. Cell Biol. 105:1473–1478.

17. Eckstein, F., D. Cassel, H. Levkovitz, M. Lowe, and Z. Seilinger. 1979. Guanosine 5′-O-(2-Thiodiphosphate). J. Biol. Chem. 254:9829–9834.

18. Honeycutt, E. J., and J. E. Niedel. 1986. Cytochalasin B enhancement of diacylglycerol response in formyl peptide-stimulated neutrophils. J. Biol. Chem. 261:15900–15905.

19. Howard, T. H., and C. O. Oresajo. 1985. The kinetics of chemotactic peptide-induced early breakdown of inositol phospholipids and Ca2+ mobilization in guinea pig neutrophils. J. Biol. Chem. 260:15771–15780.

20. Painter, R. P., K. Zahler-Bents, and R. E. Dukes. 1987. Regulation of the affinity state of the N-formylated peptide receptor of neutrophils: role of guanine nucleotide-binding proteins and the cytoskeleton. J. Cell Biol. 105:2959–2971.

21. Jesaitis, A. J., J. R. Naemura, L. A. Sklar, C. G. Cochrane, and R. G. Painter. 1984. Rapid modulation of N-formyl peptidase receptors on the surface of human granulocytes: formation of a high-affinity ligand-receptor complexes in transient association with cytoskeleton. J. Cell Biol. 98:1378–1387.

22. Jesaitis, A. J., J. O. Tolley, R. G. Painter, L. A. Sklar, and C. G. Cochrane. 1985. Membrane-cytoskeleton interaction and the regulation of chemotactic peptide-induced activation of human granulocytes: the effects of dihydrocytochalasin B. J. Cell. Biochem. 27:241–253.

23. Jesaitis, A. J., J. O. Tolley, and R. A. Allen. 1986. Receptor-cytoskeleton interactions and membrane traffic may regulate chemoattractant-induced superoxide production in human granulocytes. J. Biol. Chem. 261:13662–13669.

24. Jesaitis, A. J., G. M. Bokoch, J. O. Tolley, and R. A. Allen. 1988. Lateral segregation of neutrophil chemotactic receptors into actin- and fodrin-rich plasma membrane microdomains depleted in guanyl nucleotide regulatory proteins. J. Cell Biol. 107:921–928.

25. Kataoka, T., and M. Ut. 1982. ADP ribosylation of the specific membrane protein of C6 cells by islet-activating protein associated with modification of adenosyl cyclase activity. J. Biol. Chem. 257:7210–7216.

26. Krause, K.-H., W. Schlegel, C. B. Wollheim, T. Andersson, F. A. Waldvogel, and P. D. Lew. 1985. Chemotactic peptide activation of human neutrophils and HL60 cells: pertussis toxin reveals correlation between inositol 1,4,5-trisphosphate generation, Ca2+ transients, and cellular activation. J. Clin. Invest. 76:1348–1354.

27. Lacy, B. E., and C. B. Underhill. 1987. The hyaluronate receptor is associated with actin filaments. J. Cell Biol. 105:1395–1404.

28. Lindroth, M., P. B. Bell, and B.-A. Fredriksson. 1988. Comparison of the effects of critical point-drying and freeze-drying on cytoskeletons and microtubules. J. Microsc. (Oxf.). 151:103–114.

29. Milligan, G., J. Mullaney, C. G. Unson, L. Marshall, A. M. Spiegel, and H. McDardle. 1985. GTP analogues promote release of the α subunit of the guanine nucleotide binding protein, G2, from membranes of rat gloma C6 BUI cells. Biochem. J. 254:391–396.

30. Nozokos, C. E., K. Moran, E. Dodson, and A. Phillips. 1982. Design and operation of a high efficient magnetron sputter coater. Scanning Electron Microsc. 2:907–915.

31. Nishizuka, Y. 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature (Lond.). 334:661–665.

32. Obata, H., F. Okajima, and M. Ut. 1985. Inhibition by islet-activating protein of a chemotactic peptide-induced early breakdown of inositol phospholipids and Ca2+ mobilization in guinea pig neutrophils. J. Biol. Chem. 260:15771–15780.

33. Painter, R. K., F. Zahler-Bents, and R. E. Dukes. 1987. Regulation of the affinity state of the N-formylated peptide receptor of neutrophils: role of guanine nucleotide-binding proteins and the cytoskeleton. J. Cell Biol. 105:2959–2971.

34. Stossel, T. P. 1988. The mechanical responses of white blood cells. Adv. Immunol. 32:295–342.

35. Truett, A. P., III., M. W. Verghese, S. B. Dillon, and R. Snyderman. 1984. Calcium influx stimulates a second pathway for sustained diacylglycerol production in leukocytes activated by chemotactic factor of actin association with the cytoskeleton in rabbit neutrophils: its implications for cellular regulation. J. Cell Biol. 107:921–928.

36. Wallace, P. J., R. P. Weersto, C. H. Packman, and M. A. Lichtman. 1984. Stimulation by phagocytic cells: stimulus-response coupling mechanisms. In Inflammation. J. I. Gallin, I. M. Goldstein, and R. Snyderman, editors. Raven Press, New York. 325–342.