

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) (GDP β S) 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) (5) 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). Dihydrocytochalasin B (dhCB) and fMet-Leu-Phe were both obtained from Sigma Chemical Co. (St. Louis, MO). GDP β S was purchased from Boehringer Mannheim (Mannheim, FRG). Fluorescein-phalloidin was from Molecular Probes Inc. (Eugene, OR). Pertussis toxin was from List Biological Laboratories Inc., (Campbell, CA). New England Nuclear (Boston, MA) provided fMet-Leu-[3 H]Phe and 86 Rb $^+$. The FITC-conjugated goat anti-mouse antiserum was obtained from Cappel Laboratories (Malvern, PA), and 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-containing vacutainer tubes. After sedimentation on dextran the neutrophils were isolated according to the method described by Bøyum (11). Contaminating 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 composition: 136 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO $_4$, 1.1 mM CaCl $_2$, 0.1 mM EGTA, 1.2 mM KH $_2$ PO $_4$, 5.0 mM NaHCO $_3$, 5.5 mM glucose and 20 mM Hepes, pH 7.4.

Neutrophil Permeabilization

The cells were suspended at a concentration of 10 7 /ml in a medium with the following composition: 20 mM NaCl, 100 mM KCl, 1 mM NaH $_2$ PO $_4$, 25 mM NaHCO $_3$, 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 ms each) to an electric 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 Uppsala, 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 86 Rb $^+$, trypan blue, and lactate dehydrogenase (LDH; Fig. 5). The efflux of 86 Rb $^+$ was measured by preloading the cells with 12.5 μ Ci/ml of 86 Rb $^+$ for 60 min at room temperature, after which they were permeabilized. The contents of cytosolic 86 Rb $^+$ was confirmed in a liquid scintillation counter (1217 Rack-Beta; LKB Instruments Inc., Wallac Oy, Turku, Finland). The degree of permeabilization was also estimated by trypan blue staining. Trypan blue dissolved in 0.9% NaCl (12.5 mg/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 cytosolic LDH remaining in the permeabilized cells was determined by measuring the consumption of NADH in a spectrophotometer at 340 nm for 6 min (6).

Receptor-binding Assay

Neutrophils (5.5 \times 10 6 /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:* dhCB, dihydrocytochalasin B; F-actin, filamentous actin; fMet-Leu-Phe, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; fMet-Leu-[3 H]Phe, tritiated *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; GDP β S, guanosine-5'-O-(2-thiodiphosphate); Ins(1,4,5)P $_3$, inositol 1,4,5-trisphosphate; LDH, lactate dehydrogenase; PtdIns(4,5)P $_2$, phosphatidylinositol 4,5-bisphosphate.

transferred to a water bath (37°C) for a 5-min equilibration period. Thereafter, the cells were stimulated with 20 nM fMet-Leu-[3 H]Phe in the presence or absence of 20 μ M 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 occurred in the presence of 20 μ M 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 rapidly mixed and put on ice for 10 min, after which the suspension was centrifuged for 20 s at \sim 9,000 *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 additional centrifugation was performed. To enable determination of fMet-Leu-[3 H]Phe binding to cells treated in this manner, the final pellets were resuspended in 0.5 ml solution for 18 h at room temperature, and then transferred to scintillation vials, containing 2.5 ml distilled water and 5 ml aquasol. The radioactivity was determined in a liquid scintillation counter.

Morphological Examinations

Neutrophils suspended in the calcium-containing medium (10 6 /ml), not exposed to fMet-Leu-Phe, were plated onto Formvar-coated gold grids or standard 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 concentration 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% wt/vol; pH 7.3), whereas the samples for EM were fixed in glutaraldehyde (2.5% wt/vol) in 0.1 M sucrose and 0.1 M sodiumcacodylate, pH 7.2. These fixation procedures were carried out on ice for 15 min and then for an additional 30 min at room temperature. The samples were then stored in the fixative at +4°C until further processing.

Actin Staining. The cytoskeletal preparations were rinsed in PBS (2 \times 5 min). The cover slips were then placed in a moist chamber, and incubated in a solution of Fluorescein-phalloidin (0.6 μ g/ml) in the dark for 30 min at room temperature (4). The stained preparations were then washed twice in PBS (2 \times 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 \times) 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 \times 5 min) and then in TBS-BSA (TBS containing 1% BSA; 2 \times 5 min). The cover slips were then placed in a moist chamber, and incubated with monoclonal antitubulin antibodies (2) for 45 min at 37°C. The preparations were then washed twice in TBS-BSA (2 \times 5 min) before they were placed in the moist chamber for an additional incubation with a FITC-conjugated goat anti-mouse antiserum for 45 min at 37°C. After incubation the preparations were washed twice in TBS-BSA (2 \times 5 min) and once in PBS (5 min), before they were mounted as described above. Nonspecific fluorescence could be excluded by incubating samples with mouse antiserum and FITC-conjugated goat anti-mouse antiserum. A microscope (Carl Zeiss, Inc.) equipped for fluorescence (objective 63 \times) was used for examination and photography (film: Tri-X Pan 400 developed at 800 ASA; Eastman Kodak Co., Rochester, 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 sodiumcacodylate buffer and then dipped several times in each of three different volumes of distilled water. The samples 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

argon 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.

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 (10^7 /ml) and incubated with or without pertussis toxin (450 ng/ml) for 2 h at 37°C. After incubation the cells were resuspended (5.5×10^6 /ml) and stimulated with 20 nM fMet-Leu-Phe for 30 s at 37°C. The reactions were immediately stopped by addition of ice-cold paraformaldehyde (3.2%; wt/vol). The mixtures were stored at 4°C until staining with fluorescein-phalloidin. The samples were stained with a 100 μ l mixture of fluorescein-phalloidin (0.6 μ g/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 μ l 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).

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 prepared and also exposed to a high concentration of potassium (600 mM), exhibited almost no fluorescence. Cells pretreated with dhCB (40 μ M; Fig. 1 C) before preparation of the cytoskeleton were also well stained for F-actin, although the actin network exhibited a somewhat different morphology. In addition, treatment with 40 μ M dhCB had only a marginal effect on the cellular content of F-actin ($95 \pm 1.4\%$ of controls; measured with the phalloidin method previously described). Cells that had been permeabilized and incubated with GDP β S (Fig. 1 D), to investigate a possible role of the G-protein in the association between ligand-receptor complexes and the cytoskeleton, did not reveal any major reduction in their F-actin content compared with cells treated with Triton X-100 alone (Fig. 1 A). Permeabilization rendered the cells more spherical, consequently, the cell equivalents in Fig. 1 D are much less spread out than those in Figs. 1, A-C. Fig. 2 illustrates tubulin staining of the cytoskeleton in controls (Fig. 2 a), in potassium-treated (600 mM) preparations (Fig. 2 b) and in dhCB-treated (40 μ M) preparations (Fig. 2 c). Although a minor reduction in the content of tubulin can be seen in the potassium-treated preparation (Fig. 2 b), it is by no means comparable with the total lack of F-actin content in these preparations (Fig. 1 b). Pretreatment with dhCB (40 μ M) had no obvious effect on the cellular content of tubulin (Fig. 2 c). Fig. 3 a (15,000 \times) and Fig. 3 b (40,000 \times) are scanning electron micrographs of a single representative cell treated with Triton X-100. A complex three-dimensional network of the well preserved cytoskeleton is clearly envisaged. However, in both micrographs small sheets of what are most likely membrane residues are visible.

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 $\sim 25\%$ 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 μ M) 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 $\sim 60\%$, 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 cytosolic free calcium and the generation of InsP₃, as well as the production of O₂⁻-anions, 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 β S, by repeated electric discharges (1.7 kV/cm; 150 ms each) at +4°C. After permeabilization, the efflux of ⁸⁶Rb⁺ (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 cytosolic enzyme LDH can be noticed, even after a large number of discharges, whereas the efflux of ⁸⁶Rb⁺ 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 $28 \pm 7\%$.

Effect of GDP β S on the Binding of fMet-Leu-[³H]Phe-receptor Complexes to the Cytoskeleton

Electro-permeabilized neutrophils, incubated with or with-

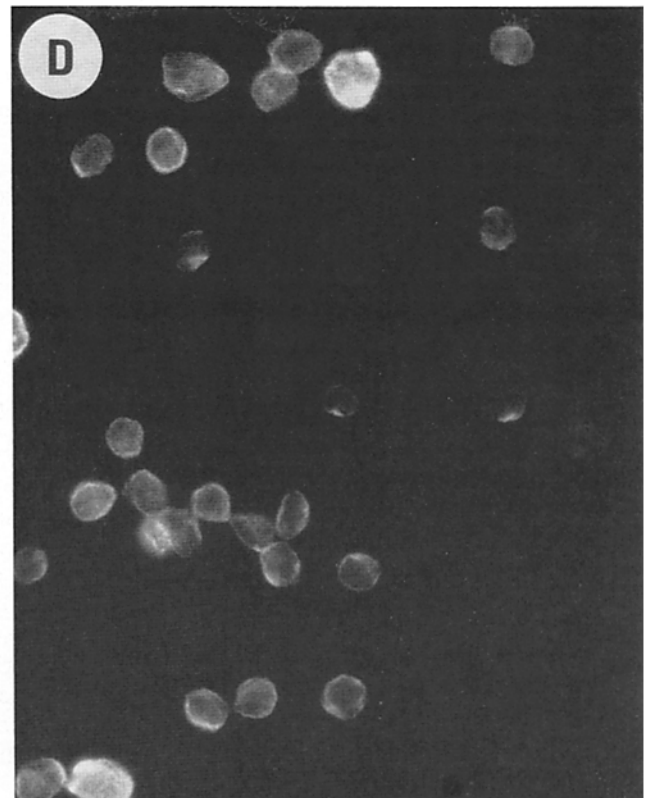
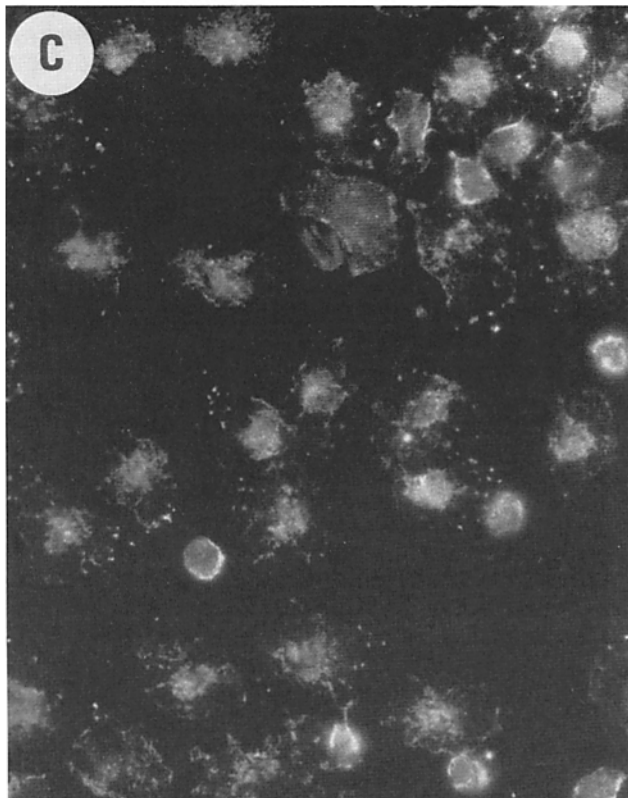
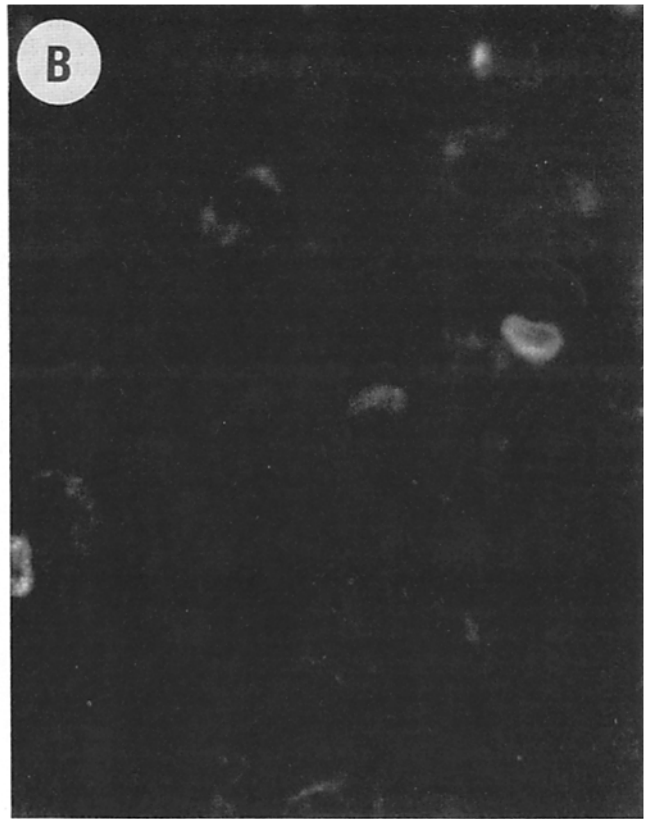
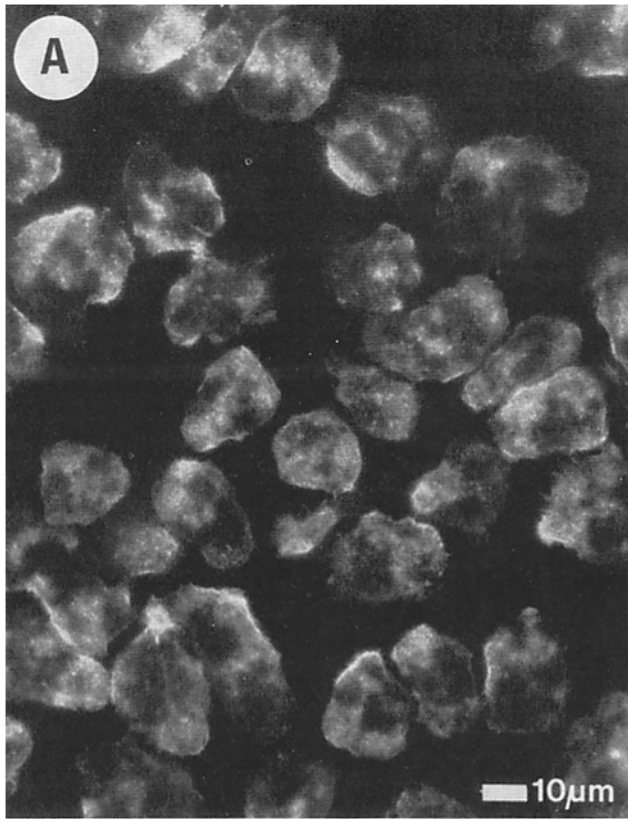


Figure 1. Photographs of cytoskeletal preparations stained with fluorescein-phalloidin. The cells were allowed to attach to standard cover slips and were then washed with Triton X-100. The obtained preparations were fixed in paraformaldehyde, after which they were stained with fluorescein-phalloidin (0.6 $\mu\text{g}/\text{ml}$). *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 dhCB (40 μM) and subsequently washed with Triton X-100 are shown in *C*. Finally, permeabilized cells incubated with GDP β S (1 mM) and subsequently washed with Triton X-100 are shown in *D*.

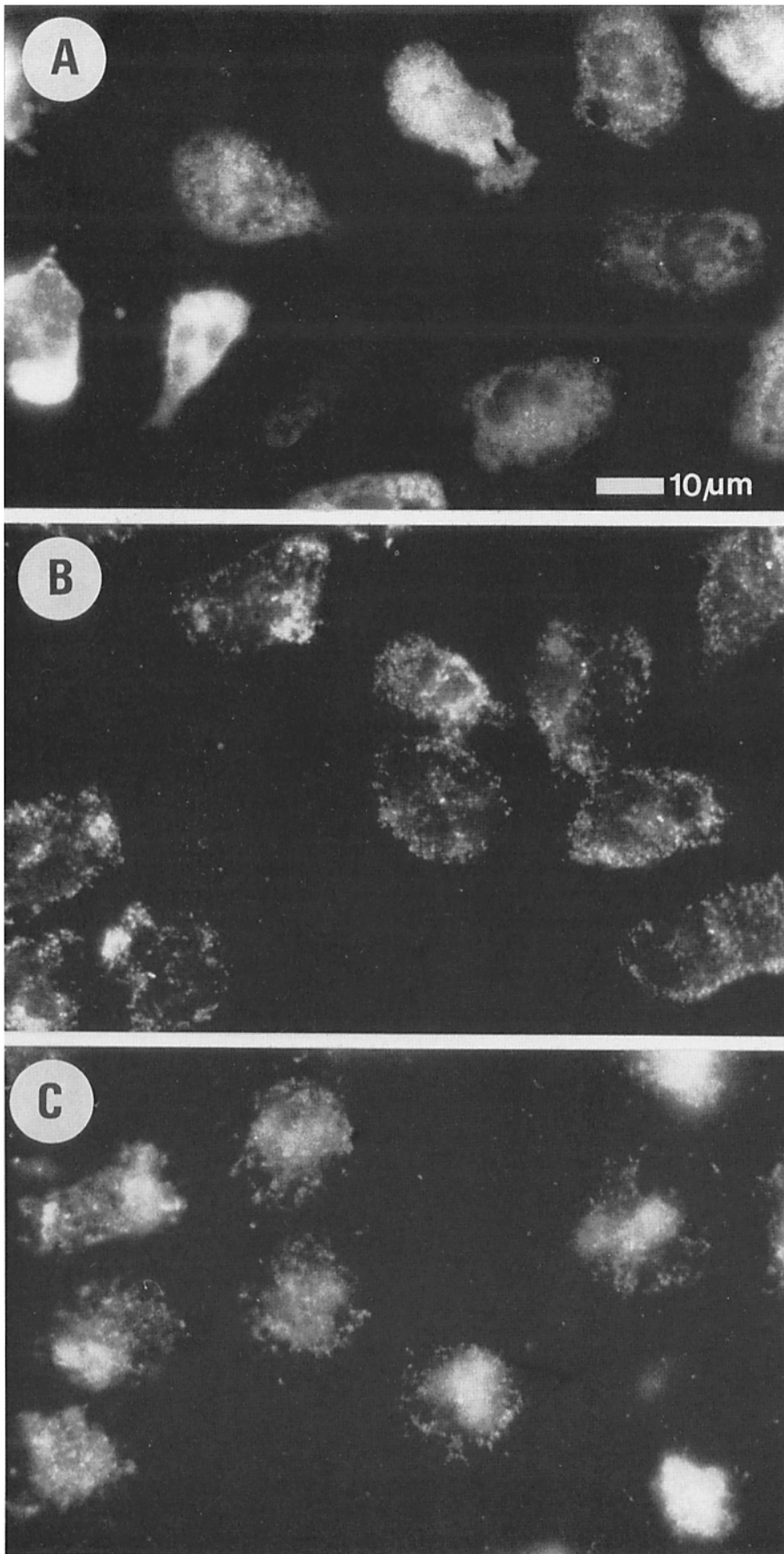


Figure 2. Photographs of cytoskeletal preparations labeled with monoclonal antitubulin antibodies and FITC-conjugated goat anti-mouse antisera. The cells were allowed to attach to standard cover slips and were then washed with Triton X-100. The obtained preparations were fixed in paraformaldehyde, after 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 dhCB (40 μ M) and subsequently washed with Triton X-100 are shown in *C*.

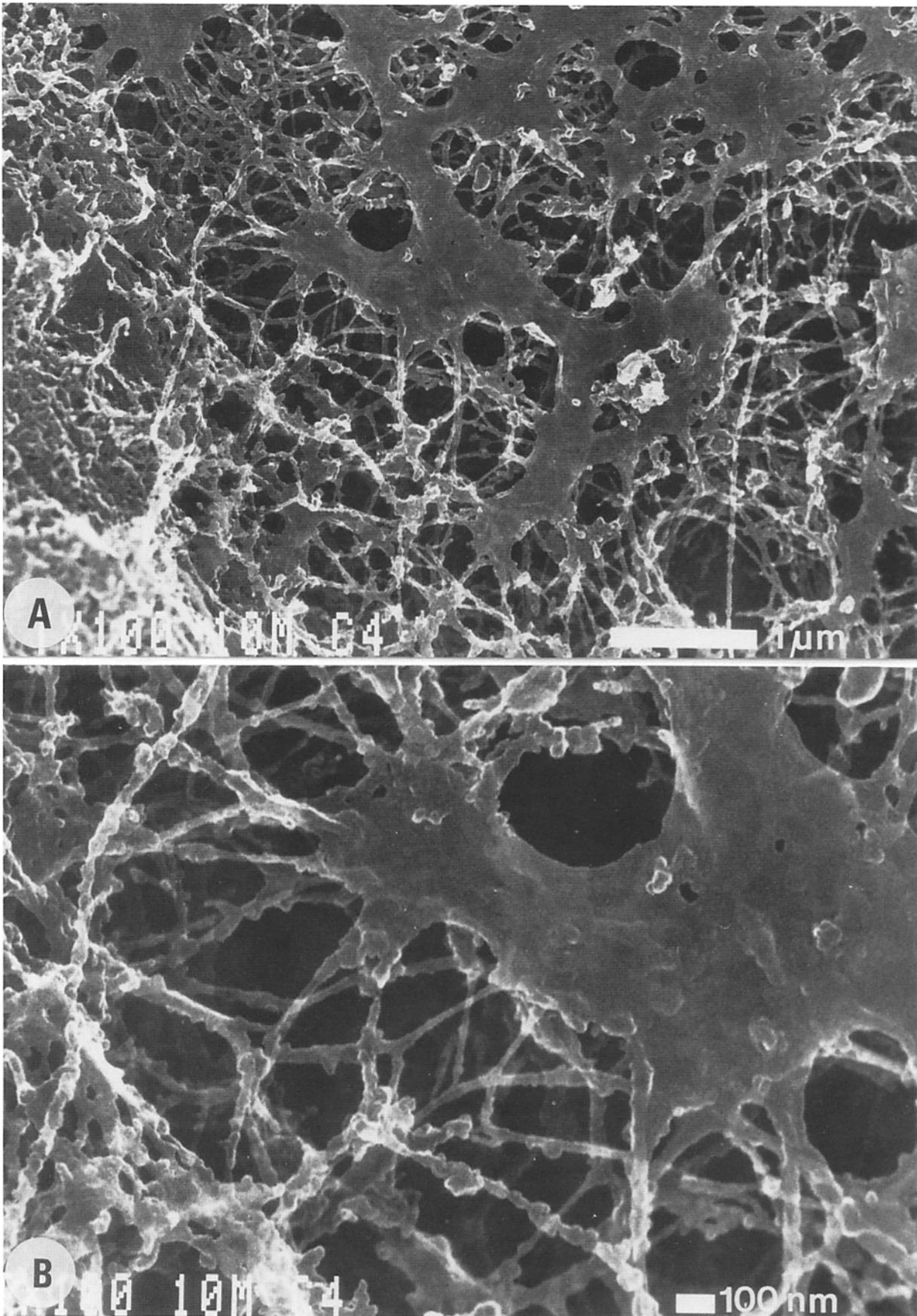


Figure 3. Electron micrographs of human neutrophils treated with ice-cold Triton X-100. The cells were allowed to attach to Formvar-coated gold grids before the ice-cold Triton-100 treatment was performed. The cytoskeletal preparations were fixed in glutaraldehyde and thereafter prepared for scanning EM. A single representative cell is shown at two different magnifications: (A) 15,000 and (B) 40,000.

Table I. Specific Binding of [³H]fMLP 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
GDPβS 1 mM	38.0 ± 6.1

Effects of potassium, dhCB, and GDPβS 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 cytoskeletal 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 GDPβS (1 mM) on the specific binding of fMet-Leu-Phe to the cytoskeleton was tested in electro-permeabilized cells. Permeabilized cells were put on ice and exposed to GDPβS 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 GDPβS (controls). The radioactivities in the control groups of the potassium, dhCB, and GDPβS experiments were calculated to $(3.4 \pm 0.3) \times 10^3$ dpm, $(1.7 \pm 0.3) \times 10^3$ dpm and $(1.8 \pm 0.2) \times 10^3$ dpm, respectively. Values in this table are mean ± SEM for $n = 5-7$.

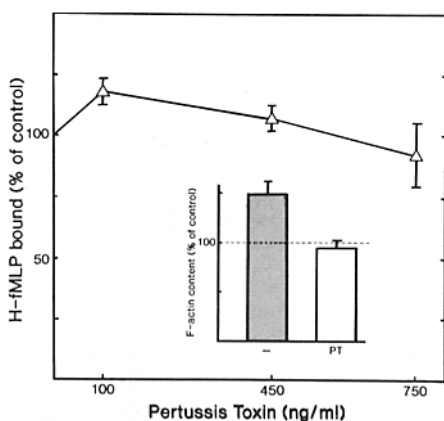


Figure 4. Effect of pertussis toxin on the subsequent binding between ligand-receptor complexes and the cytoskeletal preparation. Neutrophils were suspended in calcium-containing medium (10^7 /ml) and incubated for 2 h at 37°C with different concentrations of pertussis toxin (indicated in the figure). The cells were then washed, resuspended (5.5×10^6 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 $(3.1 \pm 0.2) \times 10^3$ 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 (5.5×10^6 /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 $n = 5$.

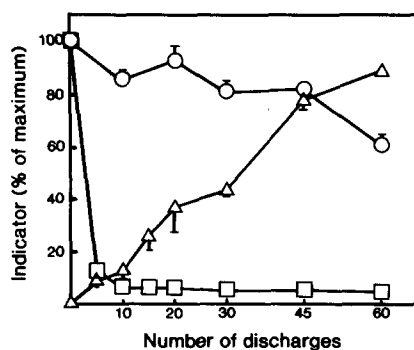


Figure 5. Electro-permeabilization of intact neutrophils. Cells were suspended in a permeabilization medium (10^7 /ml) and rendered permeable by an increasing number of exposures (150 ms each) to an electric field of 1.7 kV/cm. After permeabilization, the remaining contents of LDH (○) and ⁸⁶Rb⁺ (□) and the number of trypan blue-stained cells (Δ) were determined. The amount of LDH and ⁸⁶Rb⁺ 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 $n = 3-5$.

out GDPβS, were stimulated with fMet-Leu-[³H]Phe and thereafter exposed to ice-cold Triton X-100. The binding of fMet-Leu-[³H]Phe to the cytoskeletal preparations obtained from cells preexposed to 1 mM GDPβS was reduced by 62% (Table I).

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 the 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 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 fMet-Leu-Phe-induced polymerization of actin in neutrophils (4, 33, and data in this paper), did not reduce the binding of fMet-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 fMet-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 messenger(s). Consequently, previous and present findings suggest that the association between fMet-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 fMet-Leu-Phe-induced generation of transmembrane signals in human neutrophils via ribosylation and inactivation of the G-protein linking the fMet-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. Surprisingly enough, the present results argue against the involvement of any of the traditional transmembrane signals in this binding process. However, it was recently demonstrated that pertussis toxin did not prevent the dissociation between the α - and the $\beta\gamma$ -subunits, even if the α -subunit was efficiently ribosylated (28). Consequently, any involvement of the dissociation process itself or the $\beta\gamma$ -subunit in cellular regulation can not be adequately tested with pertussis toxin. To circumvent this problem in the present study we used GDP β S, a stable GDP analogue that competitively inhibits GTP-induced dissociation and activation of G-proteins (15, 17). When permeabilized cells were treated with GDP β S, a pronounced reduction of the associa-

tion between chemotactic factor-receptor complexes and the cytoskeleton was observed. The possibility of a nonspecific effect of GDP β S on the amount of F-actin is negligible, since no reduction in fluorescein-phalloidin staining could be detected in permeabilized cells exposed to GDP β S. Our data suggest that either the $\beta\gamma$ -subunit directly or the dissociation and release of either the α -subunit or the $\beta\gamma$ -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 $\beta\gamma$ -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 $\beta\gamma$ -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 fMet-Leu-Phe-receptor unit and the microfilamentous system in human neutrophils.

In summary, the present data demonstrate that the fMet-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 fMet-Leu-Phe transduction system most likely participates in the regulation of this binding process.

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