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Functional Molecular Complexes of Human *N*-Formyl Chemoattractant Receptors and Actin[1]

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Abstract. When human neutrophils become desensitized to formyl peptide chemoattractants, the receptors (FPR) for these peptides are converted to a high affinity, GTP-insensitive form that is associated with the Triton X-100-insoluble membrane skeleton from surface membrane domains. These domains are actin and fodrin-rich, but G protein-depleted suggesting that FPR shuttling between G protein-enriched and depleted domains may control signal transduction. To determine the molecular basis for FPR interaction with the membrane skeleton, neutrophil subcellular fractions were screened for molecules that could bind photoaffinity-radioiodinated FPR solubilized in Triton X-100. These receptors showed a propensity to bind to a 41- to 43-kDa protein band on nitrocellulose overlays of SDS-PAGE-separated cytosol and plasma membrane fractions of neutrophils. This binding, as well as FPR binding to purified neutrophil actin, was inhibited 50% by 0.6 μM free neutrophil cytosolic actin. Addition of greater than 1 μM G-actin to crude or lectin-purified Triton X-100 extracts of FPR from neutrophil membranes increased the sedimentation rate of a significant fraction of FPR two to three fold as measured by velocity sedimentation in Triton X-100-containing linear sucrose density gradients. Addition of anti-actin antibodies to FPR extracts caused a concentration-dependent immunoprecipitation of at least 65% of the FPR. More than 40% of the immunoprecipitated FPR was specifically retained on protein A affinity matrices. Membrane actin was stabilized to alkaline washing when membranes were photoaffinity labeled. Conversely, when purified neutrophil cytosolic actin was added to membranes or their digitonin extracts, after prior depletion of actin by an alkaline membrane wash, photoaffinity labeling of FPR was increased two- to fourfold with an EC_{50} of approximately 0.1 μM actin. We conclude that FPR from human neutrophils may interact with actin in membranes to form Triton X-100-stable physical complexes. These complexes can accept additional G-actin monomers to form higher order molecular complexes. Formation of FPR-actin complexes in the neutrophil may play a role in the regulation of chemoattractant-induced activation or actin polymerization. *Journal of Immunology*, 1993, 151: 5653.

Human neutrophils are charged with the important function of host defense against invasive microbes (1). To carry out this function they must seek and destroy pathogens before they cause significant damage or reproduce to uncontrollable levels. One important weapon in the neutrophil defensive arsenal is a bacterial peptide-sensing system, sensitive to *N*-formyl methionyl chemotactic peptides (2). This sensory transduction system allows neutrophils and other phagocytes to engage in directed migration toward bacteria and other sources of such peptides (3–5). The system also activates secretory and oxidative responses to chemotactic bacterial peptides.

The FPR[4] (see References 2 and 6 for a review) has recently been cloned and sequenced (7) and has been shown

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[4] Abbreviations used in this paper: FPR, *N*-formyl peptide chemoattractant receptor; GTPγS, guanosine 5′-*o*-(3-thiotriphosphate).

to be a member of the superfamily of proteins with amino acid sequence containing seven hydrophobic domains predicted to traverse the membrane bilayer (8). Transduction by this receptor proceeds via a pertussis toxin sensitive-Gi-like G protein (9, 10), which demonstrates classical negative heterotrophic effects (decrease in receptor-binding affinity) (11) upon application of GTP or its non-hydrolyzable analogs on agonist-binding affinity (12) to membranes or permeabilized cells (13).

Many of the biochemical characteristics of FPR are similar to other receptors such as the β-adrenergic and rhodopsin receptor systems whose interaction with G proteins has been extensively studied and characterized (8, 14, 15). These receptors have also been shown to interact with a 48-kDa regulatory molecule called arrestin, which has been implicated in the control of sensory transduction via its inhibitory influence on receptor/G protein coupling (16, 17). Although some evidence exists suggesting that receptor regulation may be more complex (18), the arrestins have been hypothesized to be at least partially responsible for homologous desensitization in cells containing these receptors (16, 19, 20). Desensitization of neutrophils to formyl peptide chemoattractants has been demonstrated by several laboratories both at the cellular (21, 22) and membrane level (22–24). The molecular basis for this process remains unknown although it appears that it may not be in the same class as observed for other receptor systems.

Our evidence suggests that receptor interaction with the cytoskeleton may play a role in receptor regulation and response control. We have shown that occupied FPR co-isolate with the Triton X-100-insoluble cytoskeleton of neutrophils and are converted to a high affinity, GTP-insensitive form (13, 22, 25–27). The conversion also is accompanied by loss of surface-binding activity (27) and segregation of FPR into plasma membrane domains depleted of G proteins but enriched in the cytoskeletal proteins actin and fodrin (28). These events are correlated with desensitization of cells to formyl peptides which arises from exposure of cells to formyl peptides either at 37°C (24, 29) or lower temperatures (13, 22, 29, 30). Because occupied FPR from such cells are still capable of coupling to G proteins in detergent extracts (31), it is our hypothesis that the conversion of receptors to this cytoskeletal high affinity form may represent an important step in the termination of formyl peptide-induced signal transduction and homologous desensitization (25, 31, 32).

The nature of the linkage of the occupied high affinity FPR to the cytoskeleton has not yet been determined. In this report, we document association of detergent-soluble, photoaffinity-labeled and occupied FPR with neutrophil actin. We show that this association is functional and occurs at actin concentrations compatible with a physiologic role for the association in the cell. Certain observations also suggest that the association might play a role in actin polymerization. Parts of this study have been reported in preliminary abstract form (33, 34).

Materials and Methods

Materials

Neutrophils were obtained from freshly drawn human blood and purified as described by Henson and Oades (35). Chemicals, buffers, and other preparatory materials used and membrane preparations performed were as described by Parkos et al. (36). The iodinated photoaffinity ligand, *N*-formyl-Met-Leu-Phe-N^{ϵ}[2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionyl]-[^{125}I]Lys or fMLFK-[^{125}I]ASD (formerly denoted as fMLPL-[^{125}I]SASD) used to label formyl peptide receptors was synthesized on a bimonthly basis as described by Allen et al. (37). Rabbit anti-chicken skeletal muscle actin antiserum or was obtained from ICN Biomedicals (Irvine, CA). The IgG fraction was purified (38) by affinity chromatography over protein A-Sepharose obtained from Sigma Chemical Co. (St. Louis, MO). Monoclonal mouse anti-*Amoeba proteus* actin (KJ43A, subclass IgG1) was also obtained from Sigma. Monoclonal anti-actin made against chicken gizzard actin preparations (JLA20, subclass IgM) was obtained from Oncogene Science (Uniondale, NY). Digitonin was obtained from BDH Chemicals (Poole, UK).

Radioiodination and solubilization of receptor

Human neutrophil FPR was photoaffinity radioiodinated with fMLFK-[I^{125}]ASD in isolated membranes as described previously (37). The labeled membranes were solubilized in 1% octyl glucoside (or in certain cases 1% digitonin) in 10 mM HEPES, 100 mM KCl, 10 mM NaCl, 1 mM EDTA, 10 μg/ml chymostatin, and 1.0 mM PMSF after washing them in 1 M NaCl in the same buffer or in 10 mM NaOH. GTPγS was sometimes included in the extraction buffer to prevent formation of receptor G protein complexes (39). The 100,000 × *g* supernatant of this extraction was then concentrated 10-fold in a Centricon 30 concentrator (Amicon Corp., Danvers, MA) followed by dilution in 10 volumes of the same buffer containing 0.1% Triton X-100 and in some cases concentrated again two- to 5-fold. Alternatively, the extract was passed over a wheat germ agglutinin affinity matrix and eluted in a Triton X-100 detergent-based buffer system as described previously (36).

Preparation of neutrophil cytosolic actin

Preparation of neutrophil actin was carried out following the procedure of Gordon et al. (40) with slight modifications to allow for the different starting material. Briefly, 500 ml of frozen human neutrophil cytosol obtained from the high speed supernatant fraction of a neutrophil membrane preparation as described by Parkos et al. (36) containing

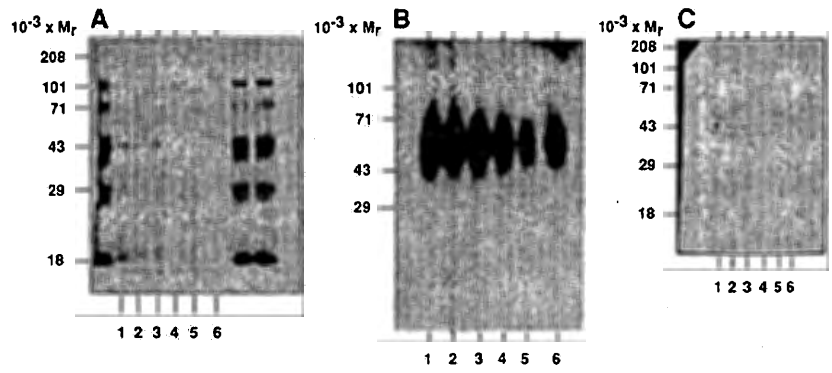


FIGURE 1. Protein composition, actin, and formyl peptide receptor content of neutrophil membranes and detergent extracts. Preparations used for the overlay and hydrodynamic analyses in this study were analyzed by SDS-PAGE with Coomassie blue staining (*A*), phosphor image analysis (*B*) of dried gel in (*A*), and Western blot analysis (*C*) using anti-actin mAb. The contents in each numbered lane are as follows: *lane 1*, membranes purified from neutrophils; *lane 2*, membranes washed with 1 M NaCl; *lane 3*, membranes washed in 10 mM NaOH; *lane 4*, digitonin extract of NaOH-washed membranes; *lane 5*, Triton X-100 extract of NaOH-washed membranes; *lane 6*, wheat germ agglutinin-purified FPR extract. Flanking lanes have m.w. standards as indicated. Each lane contains 5×10^5 cell equivalents from each type of sample.

approximately 5×10^{10} cell equivalents was thawed and dialyzed extensively (six changes over 2 to 3 days) against "buffer G" consisting of 3 mM imidazole/HCl, 0.1 mM $CaCl_2$, 0.5 mM Na_2ATP, 0.5 mM dithiothreitol, 0.02% NaN_3, pH 7.5. The dialysate was clarified by centrifugation and concentrated sevenfold (3 to 4 mg protein/ml) before loading onto a DEAE 52 ion exchange column (700 ml, 4 cm diameter). The actin in the KCl-eluted fractions was identified by SDS-PAGE and Western blot analysis, pooled, and polymerized at room temperature by the addition of 2 mM $MgCl_2$. The mixture containing F-actin was then centrifuged at 25°C for 3.5 h at 80,000 × *g* and stored as a pellet in "buffer F" (5 mM imidazole, 2 mM $MgCl_2$, 0.5 mM Na_2ATP, 0.1 mM dithiothreitol, 0.02% NaN_3, pH 7.5). Before using, the pellet was resuspended and dialyzed against G buffer for 48 to 72 h with several buffer changes and clarified by centrifugation at 100,000 × *g* for 1 h at 4°C. The state of the actin under all conditions in this study was confirmed to be in the G form or as oligomers (<20 to 25%) between 4 and 11S as determined by sedimentation analysis (not shown).

Receptor and actin contents of different preparations

Actin content of membranes, detergent extracts, and purified preparations of actin were assayed by SDS-PAGE and Western blot analysis. FPR content was determined by quantitative autoradiography or phosphor image analysis on the similar or the same gels or blots. Figure 1 shows such an analysis of photoaffinity radioiodinated membranes, before and after treatment with 1 M NaCl, 10 mM NaOH, octyl glucoside, digitonin, and lectin-affinity column purification. Figure 1 shows that lectin affinity treatment removes actin detectable by Western blot analysis to levels below that detected after NaOH treatment without proportional loss of FPR. The SDS-PAGE analysis of the purified actin is shown in Figure 2 documenting that the preparation was more than 95% pure. At least 60% of the actin migrated as a 40-kDa species as determined by Bio-Rad P-100 size exclusion chromatography. Figure 2 also compares the selectivity of the three antibodies used to bind actin for identification and immunosedimentation purposes. Both mAb (*A* and *B*) specifically detected actin in neutrophil cytosol, membranes, and pure preparations. The polyclonal antibody recognized cytosolic actin more weakly (*D*) than the former as numerous bands resulting from increased time of development appeared. However, the recognition of actin was specific, because inclusion of 100 μg/ml actin in the primary antibody incubation step inhibited binding to only the 43-kDa band (*C*). The other bands observed with this antibody were caused by nonspecific binding of IgG as evidence by the similar pattern of staining by normal rabbit IgG (not shown). The anti-actin antibodies used, all strongly recognized chicken and rabbit skeletal muscle actin. The polyclonal anti-chicken skeletal muscle actin and anti-*Amoeba* actin mAb weakly cross-reacted with rabbit heart, chicken, and rabbit smooth muscle actin, and human platelet actin preparations (not shown).

Overlay assay

Samples from a sucrose gradient fractionation of neutrophil N_2 cavitates were solubilized in SDS, run on either 7 to 18% or 7 to 22% gradient polyacrylamide gels, and stained with Coomassie blue protein stain or transferred to nitrocellulose as described previously (28). The nitrocellulose transfers were then incubated with partially purified photoaffinity radiolabeled (37) FPR prepared by lectin affinity chromatography and eluted in Triton X-100 as described above. One milliliter of the receptor extract containing 1.6×10^5

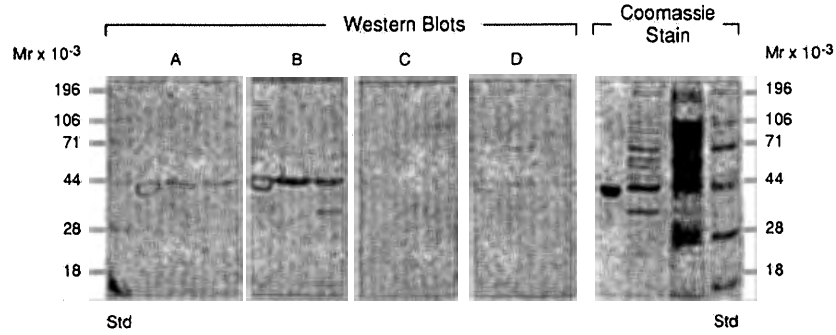


FIGURE 2. Detection of neutrophil actin by antibodies. *Outside flanking lanes* show m.w. standards as indicated. Triplet lanes of human neutrophil actin (0.4 μg, pure), *left*, cytosol (1.6×10^6 cell equivalents, 22 μg), *middle*, and membranes (8×10^6 cell equivalents, 10 μg), *right*, were analyzed by SDS-PAGE followed by Coomassie blue staining (*right-most triplet*) or Western blot analysis employing monoclonal IgG anti-*Amoeba proteus* actin (*A*), monoclonal IgM anti-chicken smooth muscle actin (*B*), and polyclonal anti-chicken skeletal muscle actin in the presence (*C*) or absence (*D*) of 100 μg/ml rabbit skeletal muscle actin.

cpm radiolabeled FPR and 1% Triton X-100 was mixed with 10 ml of Dulbecco's PBS containing 3% goat serum, 1% BSA, 0.2% Tween 20, 0.02% NaN_3 or thimerosal, and added exogenous actin as required. The "blot" was incubated with this mixture at 4°C for 40 h after which it was washed at least four times with 250 mM NaCl, 10 mM HEPES (pH 7.4), and 0.2% Tween 20 until the recovered radioactivity was less than 2% of that recovered from the incubation mixture (approximately 50% of the starting radioactivity). The blot was then dried and exposed to x-ray film to create an autoradiogram as described previously (37). Binding competition studies were plotted using Sigma Plot (Jandel Scientific, San Diego, CA) and fitted to a sigmoidal function. In certain experiments equal amounts of radioactive FPR-containing extract eluted from a blue Sepharose matrix in 2% SDS (2) was also used to test binding of denatured receptor to the nitrocellulose overlays. SDS was removed by 50-fold dilution in 1% octyl glucoside followed by concentration and exchange in 0.5% Triton X-100 as described above.

Alternatively, cytosol from a membrane preparation was run as a single lane gel (see Fig. 5) to produce strips containing the desired proteins (e.g., actin at 42 kDa) so that the overlay could be quantitated. Receptor amounts visualized by autoradiography were quantitated densitometrically using Bio-Med Instruments (Fullerton, CA) 1D/2D Laser Densitometer or a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

Velocity sedimentation and immunoaffinity isolation of FPR

A 100-μl sample of FPR in Triton X-100 extract was layered onto a 1.4-ml, 5 to 20% sucrose density gradient in 0.2% Triton X-100 in G buffer and centrifuged in a TL-100 swinging bucket rotor for 7.5 h at 53,000 rpm. In the presence of GTPγS, the receptor sediments to a depth equivalent to the sedimentation of 4S globular protein. In its absence the distribution is symmetrically shifted to a position equivalent to 6 to 7S as previously reported for receptor in octyl glucoside (31, 39), which suggests that most of the FPR is associated with G protein. GTPγS was present in some incubations and sedimentation runs to prevent receptor and G protein complexation. Sedimentation rates were calibrated against porcine cytochrome *c* (2.1S), BSA (4.4 S), porcine Ig (7.7 S), and bovine catalase (11.2S) standards as reported previously (39). Figure 3 shows that receptor retains its ability to interact with G proteins at concentrations of 0.5% Triton X-100 and lower as it is found in the 7S GTPγS-sensitive form if the nucleotide is omitted, indicating that the receptor is native and functional.

In some experiments anti-actin antisera (1/100 dilution) or its IgG fraction (4 to 40 μg/ml) were added to the FPR extracts before sedimentation to effect a sedimentation of FPR-containing immune complexes (36). Confirmation of the immune complex formation was achieved by pooling pellet fractions from such gradients, diluting them 10-fold in G buffer to reduce the residual sucrose concentration, and then passing them over a protein A-Sepharose affinity matrix. Elution was achieved after washing the column matrix in 10 volumes of G buffer, followed by pH 3.0, 0.1 M Na citrate buffer containing 0.2% Triton X-100 and 0.15 M NaCl.

Results

In previous studies, we observed that under conditions of low ionic strength but millimolar magnesium concentration, Triton X-100 could not solubilize FPR from isolated neutrophils or their plasma membranes when the cells were desensitized with [^{3}H]fMLF (26) or its photoaffinity analog (28). In octyl glucoside, however, the receptors were fully

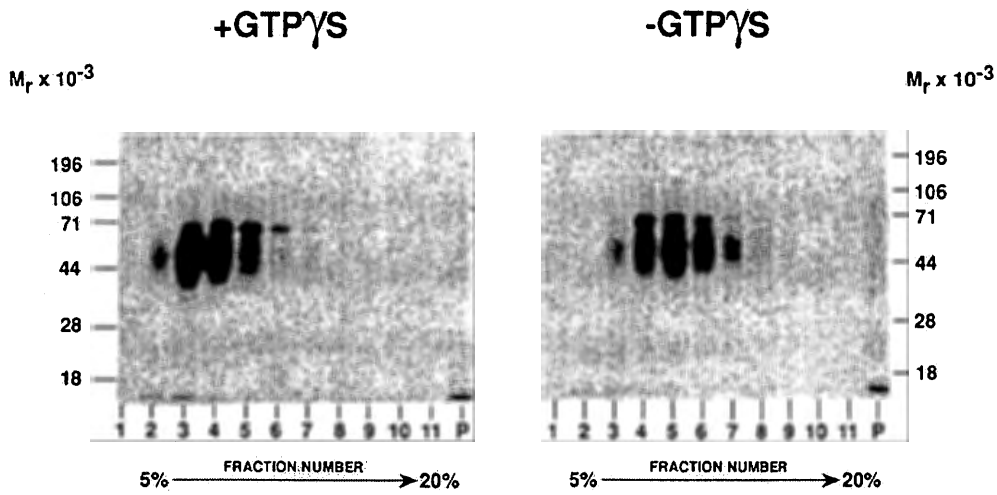


FIGURE 3. Velocity sedimentation of FPR in Triton X-100-containing sucrose density gradients. Photoaffinity-labeled FPR was solubilized from NaCl-washed membranes (*lane 3*, Figure 1) in octyl glucoside and then exchanged in Triton X-100 in G buffer with (*A*) or without (*B*) 10 μM GTPγS. The extract was then layered on 5 to 20% sucrose density gradients in G buffer and centrifuged as described in the methods section. Fractions were collected and analyzed by SDS-PAGE autoradiography (31).

extractable and could reconstitute physical complexes with G proteins (39) that retained GTP sensitivity. Because the amount of actin extracted from the membrane skeletal pellets by Triton X-100 and octyl glucoside is not significantly different (K.-N. Klotz, unpublished observations), it appeared that Triton X-100 might preserve the receptor membrane skeleton linkage and thus might be the detergent of choice to probe for detergent stable FPR cytoskeletal interactions.

Screening for FPR-binding proteins and binding to immobilized actin

One strategy used successfully in other systems to screen for proteins that might interact with cytoskeletal proteins is to apply the overlay assay method (41, 42). In our application of this procedure lectin affinity purified radioiodinated FPR in Triton X-100 was incubated with nitrocellulose transfers of neutrophil subcellular fractions separated by SDS-PAGE (Figure 4*A*). The overlays were then extensively washed, dried, and developed for autoradiography. In the developed autoradiogram shown in Figure 4*B*, a number of bands can be seen suggesting that these proteins might have a propensity for binding receptor. In the granule-enriched fractions two bands were most prominent. These had M_r values of approximately 80 kDa and 15 kDa and co-sedimented with specific, *g*, and azurophil, *a*, granules. The former corresponds to the approximate m.w. of lactoferrin, the most abundant protein in the organelle fractions of neutrophils. *Arrows* mark bands at 41 to 43 kDa observed in the heavy plasma membrane, *hp*, and cytosolic, *c*, fractions. The M_r of these bands matches that of the most abundant cytoskeletal protein, actin (28). Because this band is observed in subcellular fractions expected to contain the FPR in desensitized cells, it merited further investigation. Because the 80-kDa and 15-kDa FPR-binding proteins were evident only in the granule fractions, they were judged irrelevant to the aims of this study except for a specificity test described below.

To test the specificity of the interaction of FPR with the 41- to 43-kDa cytosolic protein, a preparative "curtain" gel, shown in Figure 5, was run with one broad lane in which only the cytosolic fraction (at one-tenth the dilution of the fraction shown in the right-most lane in Fig. 4) was electrophoresed and transferred to nitrocellulose. To conserve receptor, a strip was cut from the nitrocellulose containing the separated band and then incubated with native receptor (as above) or SDS-treated, denatured receptor. Virtually no SDS-treated receptor was retained by the strip, shown as *B*, whereas untreated native receptor shown as *A*, bound only the region containing the actin. Strips cut out from other regions of the nitrocellulose, not containing actin but equivalent amounts of other cytosolic protein (*C*), showed virtually no accumulation of radioactivity above a general background-binding level. Substitution of free fMLFK[^{125}I]-ASD for photoaffinity-labeled receptor did not produce preferential labeling of actin. Even when gels were overloaded as was the case for the initial screen, shown in Figure 4 (compare two adjacent cytosol-containing lanes), more than 50% of the radioactivity was observed in the 41- to 43-kDa band (Fig. 4*B*), which contained only 12% of the protein staining in the lane (Fig. 4*A*).

Because the concentration of FPR in the incubation buffer is estimated to be 5 orders of magnitude lower that the actin on the strip, ~100 pM vs 10 μM, it was not possible to block photoaffinity-labeled FPR with unlabeled FPR. Instead, the specificity of the binding of the FPR to

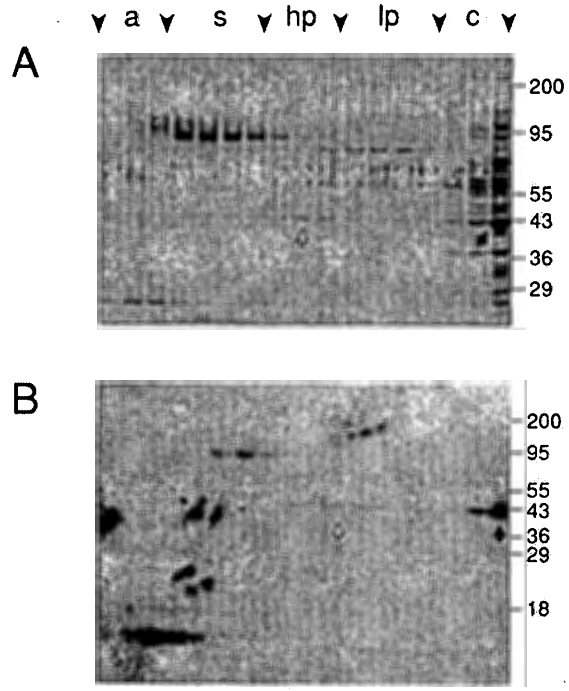


FIGURE 4. Binding of partially purified FPR to a 41- to 43-kDa protein in the heavy plasma membrane fraction of human neutrophils. Photoaffinity radioiodinated formyl peptide receptor was incubated with human neutrophil subcellular fractions that were prepared on linear sucrose density gradients, solubilized and separated by SDS-PAGE, and then electro-transferred to nitrocellulose as described in *Materials and Methods*. *A*, A stained 8 to 18% polyacrylamide gradient gel of the fractions shows the major protein components of the gradient distribution. *a*, azurophil granules; *s*, specific granules; *hp*, heavy plasma membrane; *lp*, light plasma membrane; *c*, cytosol. *Arrow* indicates the major 41- to 43-kDa protein band in the heavy plasma membrane fraction (*open*) and cytosol (*filled*). *B*, Autoradiogram of the nitrocellulose transfer of the same fractions from a 7 to 22% polyacrylamide as (*a*) above, overlaid with partially purified radioiodinated FPR, washed, and exposed to x-ray film. *Arrows* indicate binding of the FPR to a 41- to 43-kDa band in the heavy plasma membrane fraction (*open*) and cytosol (*filled*).

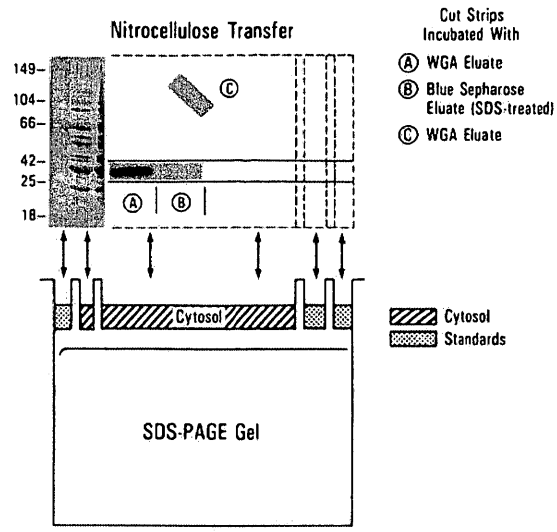


FIGURE 5. Specificity of binding of partially purified FPR to a SDS-PAGE-separated 41- to 43-kDa protein on nitrocellulose. The cytosol fraction from Figure 4 was run on a single lane "curtain" polyacrylamide gel. A strip containing the 42-kDa band was cut from this curtain and divided into pieces (*A* and *B*). An identical size piece from another region of the gel was cut out as a control (*C*). Partially purified radiolabeled FPR in 0.1% Triton X-100 was then incubated with *A* and *C* whereas SDS-treated FPR reintroduced into Triton X-100 was incubated with *B* as described in *Materials and Methods*. Native FPR but not SDS-denatured FPR is observed on the strip containing 42-kDa proteins.

the actin-containing strip was examined by including purified neutrophil cytosolic actin in the receptor incubation mixtures as is shown in Figure 6. The competition for receptor by neutrophil cytosolic actin in the liquid phase occurred at submicromolar cytosolic actin concentrations (EC_{50} = 0.6 μM). Crude cytosol or rabbit skeletal muscle actin also inhibited the binding of receptor to the strips in a comparable actin concentration range (rabbit actin was more efficient with an $EC_{50} \approx 0.1$ μM, not shown). BSA or OVA at similar or higher concentrations (3 mg/ml) did not interfere with the binding of the receptor to the immobilized actin. Radioactivity bound to the band was linear with actin bound to the nitrocellulose from 0 to 40 μg/ml actin (not shown). A similar examination of the radioactivity bound to the lactoferrin-containing band of the specific granule fractions, *s*, in Figure 4 showed that no radioactivity was lost as a function of increasing soluble actin concentration, suggesting that the inhibition of FPR binding to the 41- to 43-kDa band was indeed specific. We conclude that the solubilized FPR was probably binding to the most abundant species in this band, actin. Binding to the low m.w. band in the azurophil granule-containing fractions (*a*) may or may not be specific and deserves further attention. It was not examined further in these studies.

FPR binding to soluble actin

The above results suggested that at least some of the solubilized receptor was interacting with the immobilized actin on nitrocellulose. Even though the assay is performed in Triton X-100 with overwhelming amounts of BSA and goat serum in the buffer, it is possible that nonspecific interactions are the cause of this interaction. Thus, an alternative method to confirm the interaction of FPR with actin was required. Sedimentation analysis of FPR was chosen as an alternative method because it also provides an indication of the size of the molecular complexes formed and because the method has been used by our laboratory to detect GTP-sensitive reconstituted complexes of receptors and G proteins in octyl glucoside and Triton X-100 (Fig. 2) (39).

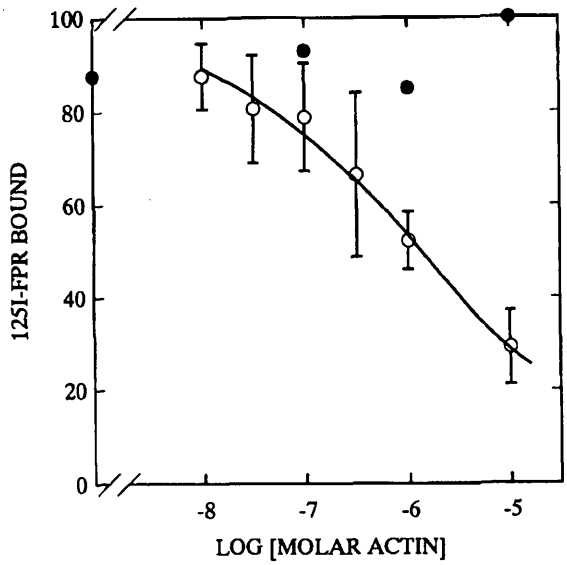


FIGURE 6. Specificity of FPR binding to nitrocellulose-bound 41- to 43-kDa protein of neutrophil cytosol. *Vertical strips* parallel to the direction of migration were excised from curtain gels run identically as described in Figure 5 and *Materials and Methods*. Sample amounts run were cytosol, 6 μg, and specific granules, 10 μg. These were incubated with lectin-purified receptor in the presence of different concentrations of purified human neutrophil cytosolic actin. After extensive washing, the strips were dried and developed for autoradiography and the amount of radioactivity bound to each actin-containing band was quantitated. The percent of the maximum activity (100% with no added actin) is plotted against the log of the micromolar concentration of soluble actin added to the incubation mixture. Each data point for the cytosol experiment (○) represents the average of three independent determinations with *error bars* indicating the SEM. The *solid line* is a nonlinear least squares fit of a sigmoidal curve (see *Materials and Methods*) to this data from which the EC_{50} was determined to be 0.6 μM. Binding of FPR to the 80-kDa band of the specific granules (●) was accurate to 10 to 15% based on two independent measurements.

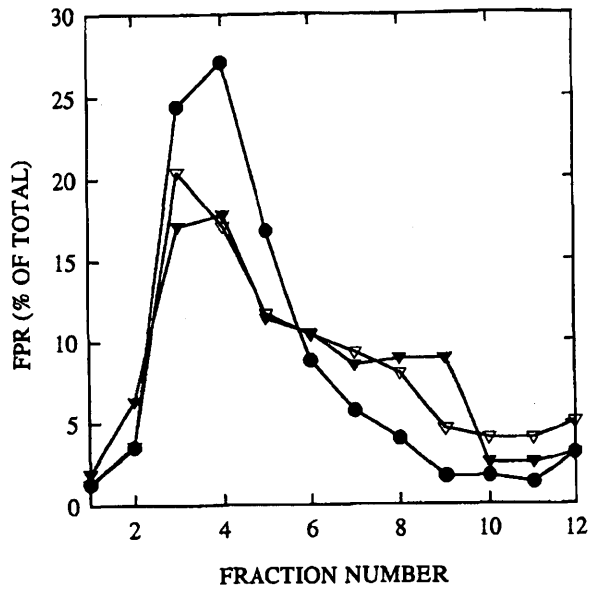
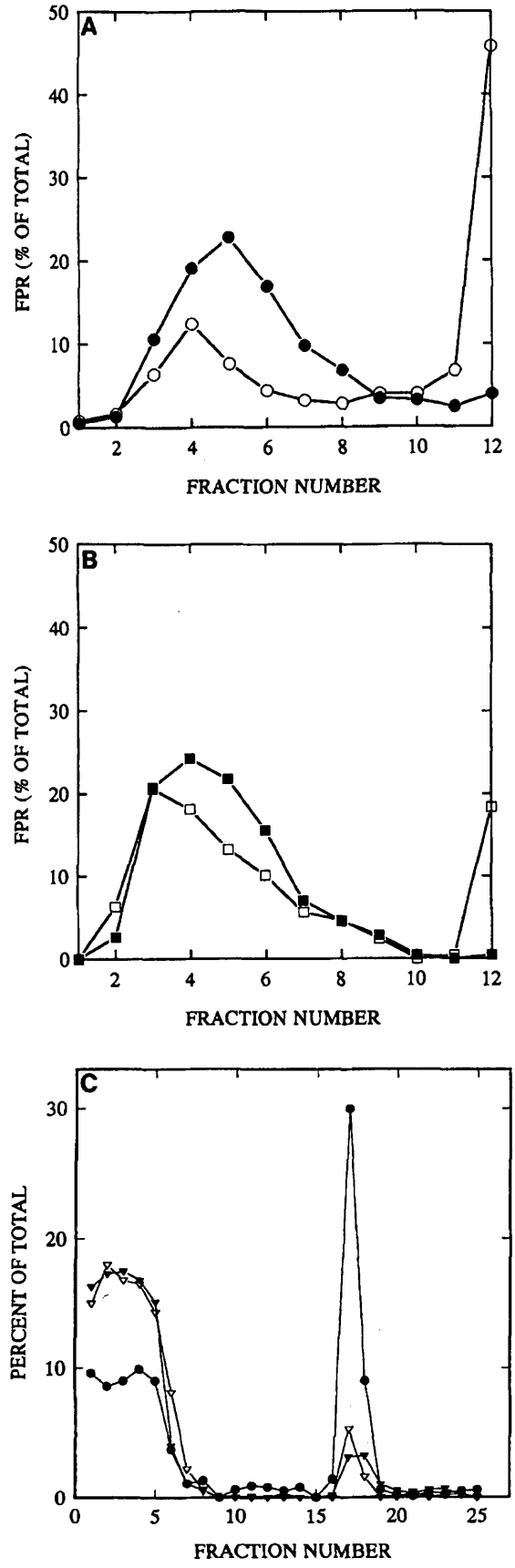


FIGURE 7. Actin increases sedimentation rate of soluble FPR. FPR, partially purified by lectin affinity chromatography and maintained in Triton X-100 in G-buffer, was preincubated without (●——●) or with 0.25 mg/ml (6 μM, ▽——▽) or 2 mg/ml (47 μM, ▼——▼) purified cytosolic G-actin as described in *Materials and Methods*. The mixture was layered on top of a linear sucrose density gradient containing 0.2% Triton X-100, and centrifuged 7.5 h at 53,000 rpm in a Beckman TLS 55 rotor (100,000 × *g*) to effect significant receptor sedimentation. Receptor content of each fraction was quantitated by densitometric scans of autoradiograms or phosphor image analysis of SDS-PAGE separated receptor and was plotted as a function of fraction number. Fraction 1 contained 5% sucrose and fraction 12 contained 20% sucrose (one of four experiments).

Thus, the lectin-purified radioiodinated FPR was incubated with different concentrations of purified neutrophil cytosolic actin and then sedimented in Triton X-100-containing sucrose gradients in G buffer. Figure 7 shows that the sedimentation rate of approximately 20% of the FPR increased after an overnight exposure to neutrophil cytosolic actin. Similar results were obtained when the incubation with actin was performed for 2 h. The interactions were also observed, but to a lesser degree, when cruder preparations of FPR were used, in which the lectin affinity step was omitted (not shown). Examination of the sedimentation of the actin in these gradients (not shown) indicates that most of the actin sediments at a rate compatible with monomers. There was no evidence of large actin networks that might trap FPR and thus cause a nonspecific sedimentation. Why only a percentage of receptors interact with actin is still under study.

Immunosedimentation of FPR with anti-actin antibodies

To prove that the complexes formed do indeed contain actin, it would be necessary to immunoprecipitate FPR with anti-actin antibodies. However, under the conditions of the above experiments, a negative result would be inconclusive because so much exogenous actin is added that uncomplexed actin would compete with FPR-complexed actin. On the other hand, the fact that only 20% of the lectin-purified FPR was affected by addition of actin suggested that, in the actin-rich environment of the cell, occupied receptors that were not associated with G-proteins might already be associated with cytoskeletal proteins that promote the formation of the very high affinity complexes we and others have observed (31). Thus, we tested three different anti-actin antibodies for the ability to immunoprecipitate FPR from crude and lectin-purified extracts of FPR. Figure 8



shows that the polyclonal anti-chicken back muscle and anti-Amoeba cytoplasmic actin mAb caused a significant immunoprecipitation of the FPR from both types of preparations. More than 45 ± 5% (n = 4) of the lectin-purified FPR and 21 ± 3% of the FPR in the crude extract were found in the pellet fractions of the gradients containing the polyclonal rabbit anti-chick actin antibody. The values for the anti-Amoeba actin were 20 ± 3% and 10 ± 4%, respectively. Addition of normal rabbit serum, normal rabbit IgG, irrelevant isotype-matched mAb, or IgM anti-actin mAb (JLA20) resulted in no significant receptor accumulation in the pellet fraction.

To prove that the pelleted receptor was indeed part of a specific immune complex, the pellet fractions were pooled and passed over a protein A-Sepharose affinity matrix. Figure 8*C* shows that more that 40% of the radioactivity was retained by the matrix and that it could be eluted with a low pH. When either normal IgG or pure actin was added (100 μg/ml) to the mixture before passing over the column, less than 1% of the radioactivity was retained confirming the specificity and reversibility of the antibody interaction. Because the FPR-actin complexes bound to the antibodies are very stable, requiring SDS to achieve dissociation, the competition probably represents exchange of actin for FPR-actin complexes on the antibodies. High affinity antibodies demonstrating low avidity would thus account for the seemingly partial interactions observed.

The low avididity nature of the antibodies was confirmed when the fractions containing the unpelleted receptor were pooled, concentrated, reincubated with anti-actin antibody, and then resedimented. Between 25% and 35% of the originally unpelleted pool was now pelleted. The FPR in the unpelleted pool also showed no ability to interact with actin

FIGURE 8. Immune complex formation of FPR with anti-actin antibodies. Triton X-100-soluble FPR, purified by lectin affinity chromatography, was incubated for 16 h at 4°C with *A*) 1/100 diluted rabbit anti-chicken back muscle actin antisera (○——○) or normal rabbit antisera (●——●); *B*) monoclonal anti-*Amoeba proteus* actin antibody (□——□) or control irrelevant, isotype-matched mAb (■——■) and sedimented in a Triton X-100-containing linear sucrose gradient as in Figure 3 or 7. Fractions were collected including pellet fraction (no. 12) and solubilized in SDS and then analyzed by SDS-PAGE autoradiography for the presence of receptor. Percent of the total receptor content in the gradient, determined by densitometric evaluation of autoradiograms of the fractionation, are shown with the top of the gradient to the left (5% sucrose) and the pellet fraction to the right (20% sucrose). *C*) pellet fractions of *A* were pooled and passed over a protein A-Sepharose affinity matrix (●) or mixed with 100 μg/ml rabbit skeletal muscle actin (▼) or 100 μg/ml normal rabbit IgG (▽) and then passed over the affinity matrix. Elution was carried out with pH 4 citrate buffer (fractions 15–25) after washing with G buffer (fractions 9–14) as described in *Materials and Methods*. One of two experiments.

directly, failing to shift FPR sedimentation to higher rates as was shown in Figure 7. These results suggest that more than 55 to 65% of FPR in the detergent extracts exists in preformed complexes with actin and the binding of a significant fraction of the complexes to exogenously added actin probably occurs via actin addition to the complex.

Promotion of photoaffinity labeling of FPR by actin

To demonstrate the physiologic relevance of the actin-associated FPR, it would be useful to compare the sedimentation of the occupied and unoccupied receptors in the presence and absence of exogenously added actin. However, our attempts to cleave and free the photoaffinity-labeled FPR of the disulfide-linked fMLFK has so far been unsuccessful. Attempts at freeing actin associated with photoaffinity-labeled FPR without denaturing the receptor also have not been successful. These observations suggest that occupied receptor must be very tightly coupled to actin and that perhaps unoccupied receptor without actin would have a reduced affinity for its ligand analogous to that observed when receptor is uncoupled from G proteins. Such a reduced affinity might be reflected in a reduced efficiency of photoaffinity labeling. To explore this possibility, we examined the effects of actin on the photoaffinity labeling of the receptor with fMLFK-[^{125}I]ASD. Actin-depleted membranes were prepared by sonication of neutrophil membranes with 10 mM NaOH (43). It is noteworthy that these membranes were depleted of measurable actin (not shown) in contrast to the membranes that were prelabeled with the photoaffinity label (Fig. 1). The actin-stripped membranes were then incubated with 5 nM fMLFK-[^{125}I]ASD in the presence and absence of 1 μM non-radioactive fMLFK-ASD with or without 1 μM purified neutrophil actin. In Figure 9*A* (*left*), it can be clearly seen that receptor labeling is significantly increased in the presence of exogenously added actin. No such enhancement was observed in the nonspecifically labeled intracellular 68-kDa protein or if equal concentrations of OVA were included in the labeling mixture instead of actin (not shown).

It is possible, that actin addition to actin-depleted membranes somehow affects the state of the membrane promoting increased photoaffinity labeling. To remove membrane effects, the membranes were first solubilized in 1% digitonin, a detergent that preserves FPR binding activity (44), and then labeled in the presence or absence of actin. The right panel of Figure 9*A* (*right*) shows that the difference is also clearly evident, suggesting that indeed receptor actin interaction promotes increased photoaffinity labeling. To quantitate the effect of actin on photoaffinity labeling, the labeling dependence on actin concentration in digitonin was determined. Figure 9*B* shows that actin is 50% effective in enhancing the photoaffinity labeling of the

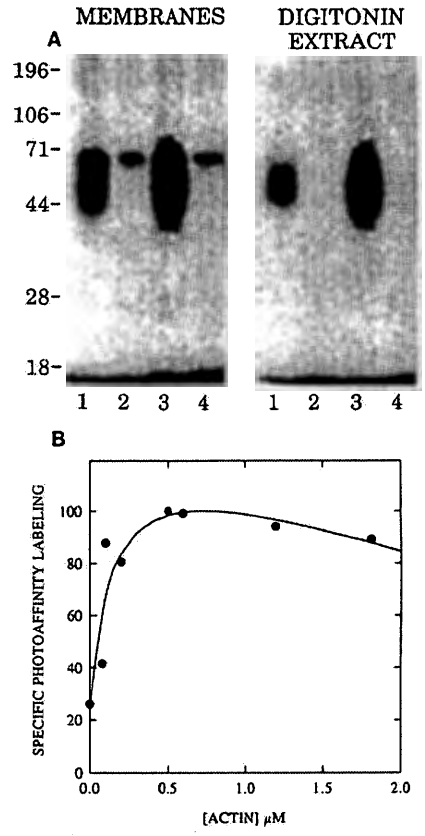


FIGURE 9. Effect of actin on the photoaffinity radiolabeling of FPR. Neutrophil membranes were stripped of actin by treatment with 10 mM NaOH for 10 min, washed in buffer, and either radiolabeled directly or after preparation of a digitonin extract of membranes in the presence and absence of up to 1.2 μM of purified neutrophil cytosolic G-actin. *A*, autoradiographic comparison of receptor labeling shows an increase in photoaffinity labeling after the addition of actin, lanes 3 and 4 as compared with labeling with no actin added (*lanes 1 and 2*). *Lanes 2* and *4* show extent of nonspecific radiolabeling in the presence of 1.0 μM non-radioactive photoaffinity analog. *B*, the dependence of the photoaffinity labeling, quantitated densitometrically, is plotted as a function of the actin added. *Each point* represents the average of 3 runs and is given as a percent of the maximal labeling.

receptor at a concentration of approximately 0.1 μM. This value agrees reasonably well with the mid-point (0.6 μM) determined for the soluble FPR binding to actin in Triton X-100 in Figure 6, even though a very broad concentration dependence was observed in the latter experiment. The binding to FPR in Triton X-100 is clearly saturable by 5 μM as is shown in Figure 7. Thus, the anomalously broad dependence was probably the result of the unusual state of the actin on the nitrocellulose resulting from electrophoretic transfer from SDS. Together all these results supports the hypothesis that actin interacts with receptor and that the interaction may be direct enough to promote agonist binding to the FPR.

Discussion

The purpose of the experiments described in this work was to determine the molecular basis for the interaction of FPR with the membrane skeleton of human neutrophils. This determination was made by attempting to identify a protein component of the subsurface membrane skeleton of human neutrophils that interacts with detergent solubilized FPR. Our evidence suggests that this component may be actin. In brief, this evidence rests on the ability of occupied photoaffinity-labeled FPR in Triton X-100 extracts to: 1) bind to SDS-denatured actin electro-transferred to nitrocellulose membranes; 2) to associate with native actin in detergent solution; and 3) to be immunoprecipitated with polyclonal anti-chicken skeletal muscle and anti-Amoeba actin mAb. In addition, because actin also appears to enhance the photoaffinity labeling of FPR in actin-stripped membranes or the digitonin extracts of these membranes, the interaction of the solubilized FPR may not only be direct and physical but also functional.

Although actin has been described as being a "sticky" protein, which could conceivably generate artifactual interactions with hydrophobic membrane proteins (45), this possibility is unlikely. The actin/FPR associations measured occurred at Triton X-100 concentrations (0.1 to 0.5%), well above the critical micelle concentration (0.016%). Inclusion of detergent would minimize hydrophobic interactions between proteins of the sort measured between actin and albumin or cytochrome *c* observed in physiologic salt solution without detergent (45). Inclusion of the high concentrations of BSA (1%) and goat serum (3%) in the detergent-containing nitrocellulose overlay assays also would act to block nonspecific hydrophobic interactions.

Specificity is also supported by the fact that a) binding to the actin band in the overlay assay is competed by soluble actin, b) demonstrable FPR/actin complexes form in solution that are detectable by sedimentation analysis, c) SDS abolishes the ability of the receptor to form complexes, d) binding to at least one of the non-actin bands in the overlay assay of the cell is not competed by actin in the incubation mixture, and e) complexes are not detectable in sedimentation or overlay analyses when even greater concentrations of other proteins such as irrelevant Ig, BSA, or OVA are used in the incubation mixture. Thus, although, it is impossible to perform a competitive "blocking" experiment with excess nonradioactive FPR because of limitations in FPR material available, these observations strongly suggest that the interaction is indeed specific.

Our additional attempts to prove specificity of the interaction with anti-actin antisera resulted in the discovery that at least 65% of 4S FPR in Triton X-100 already appears to be complexed with actin or an immunologically related protein. This is supported by our previous hydrodynamic studies that indicated that the receptor was highly hydrophobic with a partial specific volume 0.88 ml/g (46) characteristic of integral membrane proteins with a significant transmembrane aspect. For such a protein, 4S would be an anomalous sedimentation coefficient and would be indicative of a higher order molecular complex of approximately 100 to 150 kDa. This size is compatible with FPR actin complexes. Thus, the fact that the immunosedimentable 4S FPR can bind additional actin suggests that it may participate in the actin polymerization process allowing actin addition to the complex. We are currently attempting to determine why only a minor (20 to 30%) fraction of the Triton X-100-solubilized FPR binds the additional actin.

In the absence of GTPγS, the 4S FPR-actin complexes are fully capable of interacting with endogenous (see Fig. 1) or exogenous (D. Siemsen, R. Bommakanti, and A. Jesaitis, unpublished observations) G protein to form physical complexes of apparent sedimentation coefficients of approximately 7S in analogous fashion to complex formation already reported in octyl glucoside (31, 39). In fact, in order to carry out the experiments described herein, GTPγS was usually included in the buffers to restrict the analysis to the G protein-free, 4S FPR form. These observations imply that either actin dissociates from the FPR to allow G protein to complex with the receptor or that it is retained in a complex of the receptor and G protein. Our preliminary qualitative evidence suggests that anti-actin antiserum still mediates some FPR immunoprecipitation in extracts not treated with GTPγS and displaying the 7S FPR sedimentation profile (D. Siemsen and A. Jesaitis, unpublished observations). However, precisely what percentage of the immunoprecipitated complex contains G protein is still undetermined.

Either of the above possibilities, however, may afford a number of new and interesting insights into the functioning of FPR, and possibly, other receptors. The latter possibility is that occupied-FPR/G protein/actin complexation might not only trigger release of the G protein α-subunit but also provide a platform or nucleus for actin polymerization directly from a FPR/actin complex. The former alternative, that the receptor exchanges actin for G protein and vice versa, is also intriguing as it provides for a mechanism of receptor sequestration and removal from G protein-rich membrane microdomains as well as providing for potential sites for actin filament nucleation and growth. Such a hypothesis would be compatible with a) the very rapid kinetics of the actin polymerization response (47), b) sensitivity of the response to pertussis toxin (48) and cytochalasin B (47, 49), c) a regulatory role for the process by providing a mechanism for removal of further interaction with G proteins, d) requirements for establishment of stimulated microfilament growth for cell polarity and chemotaxis (50), and e) the structural similarities between the two proteins mentioned above.

Membrane receptor interactions with actin are not unique

FPR	322	R	A	L	T	E	D		S	T	Q	T	S	D	T	A	T
VINCULIN	433	R	S	L	G	E	I		S	A	L	T	S	K	L	A	D
CORONIN	247	R	A	F	T	T	P	L	S	A	Q	V	V	D	S	A	S

FIGURE 10. Comparison of the amino acid sequence of the carboxyl-terminal tail of FPR with regions of coronin and vinculin demonstrating unusual identity (*shaded*). The amino-terminal arginine of each peptide region is identified with a number (*left box*) that corresponds to its position in the sequence of each protein.

to the FPR system in neutrophils. In human platelets there appears to be a direct linkage of GPIIb and III to actin in the cytoskeleton after stimulation of the cell with Con A (51, 52). The acetylcholine receptor co-purifies with actin (53, 54) as well as with another 43-kDa protein that organizes clusters of these receptors in muscle (54). The 67-kDa laminin receptor, important in metastatic invasion, appears also to be an actin-binding protein (50, 55) that anchors this receptor to the cytoskeleton. Most recently, the epidermal growth factor receptor has been shown to have a direct interaction with actin through an 8-amino acid stretch of its sequence, having only 62% identity with profilin (56). It is noteworthy that the investigators concluded this sequence to be the *only* stretch that mediated epidermal growth factor receptor binding to actin, adding further significance to the longer stretches of sequence identity between FPR and actin-binding proteins (see below).

Sequence comparison of these and other actin-binding proteins with cytoplasmic domains of FPR shows little sequence similarity except for a short stretch of 15 amino acids on the carboxyl terminal tail of the receptor. Figure 10 shows some noteworthy features of such a region displaying 40 to 50% identity to coronin (57) and vinculin (58), suggesting that the interaction of these proteins with actin might provide some clues about the actin interactions of FPR. It is of further interest that this region of the receptor has been shown to be important in FPR complexation with G_i (59), and that G proteins may contribute to the formation of cytoskeletal structures (60–62).

It must be remembered that actin is very abundant in the cytoplasm with the concentration of the unpolymerized G form estimated to be 100 μM (63) depending on the stimulation state of the cell. Free G-actin is probably less abundant, but in the micromolar range, given that profilin (64, 65), β4 thymosin (66), and other proteins may sequester a significant fraction of actin subunits (67). Because our measured interaction of solubilized FPR appears to be in the submicromolar range, then any occupied receptors would rapidly bind available G-actin having specific sites available for receptor. This interaction coupled with observed receptor clustering (68, 69) and actin polymerization might then lock receptor into its high affinity, effectively nondissociating form. Results from another study, in which we examined complexes of receptors extracted directly with Triton X-100 from isolated membrane skeletons, supports this hypothesis. They suggest that the receptor membrane skeletal interaction that functionally correlates with the desensitization of the cell depends on the integrity of the polymerized actin in the membrane skeleton.[5]

In summary, our results support the hypothesis that actin physically and functionally interacts with the formyl peptide. Numerous speculative possibilities exist for functional roles of FPR actin interaction some of which may have direct bearing on the molecular basis of chemotaxis and receptor control.

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