Lateral Segregation of Neutrophil Chemotactic Receptors into Actin- and Fodrin-rich Plasma Membrane Microdomains Depleted in Guanyl Nucleotide Regulatory Proteins

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Abstract. Subcellular fractions were prepared from human neutrophils desensitized at 15°C with stimulatory doses of the photoaffinity derivative *F*-Met-Leu-Phe-N^{ϵ}-(2-(ρ-azido[^{125}I]salicylamido)ethyl-1,3′-dithiopropionyl)-Lys. The covalently labeled receptors were found in a membrane fraction of higher density than those from cells preexposed to ligand at 4°C but not desensitized. The denser fraction (ρ ≅ 1.155 g/cc) was the cellular locus of the membrane associated cytoskeletal proteins, actin, and fodrin, as detected immunologically on western blots. The light fraction (ρ ≅ 1.135), cosedimented with neutrophil plasma membrane markers, plasma membrane guanyl nucleotide regulatory proteins, and several characteristic polypeptides identified by SDS–PAGE, including a major 72-kD species. The photoaffinity-labeled species in either case showed the same mobility on SDS–PAGE (M_r = 50,000–70,000) corresponding to previously reported values for *N*-formyl chemotactic receptors. These labeled receptors were sensitive to proteolysis after exposure of the intact photoaffinity-labeled cells to papain at 4°C. We conclude that (*a*) the fractions isolated are probably derived from different lateral microdomains of the surface of human neutrophils; (*b*) the higher density fraction contains occupied *N*-formyl-chemotactic receptors previously shown to have been converted, to a high affinity, slowly dissociating form coisolating with neutrophil cytoskeleton and implicated in the termination of formyl peptide–induced neutrophil activation; and (*c*) the translocation of receptors to these microdomains may serve to compartmentalize receptors and perhaps regulate the interaction of the receptor/G-protein transduction pair.

HUMAN neutrophils play a central role in host defense and serve as a model system for the study of the sensory transduction in ameboid cells. In studies of the sensory transduction processes of the human neutrophil, we and others have shown that shortly after occupancy at 37°C, *N*-formyl chemotactic peptide receptors are converted to a high affinity, slowly dissociating form (16, 17, 29, 35), found to coisolate (16, 17, 35) transiently with the Triton X-100–insoluble cytoskeleton of the cell. In our studies, we suggested that the coisolation of cell surface ligand-receptor complexes with the Triton X-100–insoluble fraction was the manifestation of a steady-state process driven by association of newly occupied receptors with the cytoskeleton followed by their removal by endocytosis (16). We also found that we could first occupy the receptors at the cell surface at lowered temperatures (15°C) avoiding internalization (31) and response (21) and thus trap them into the high affinity state coisolating with the cytoskeleton. Rapidly warming such cells back to 37°C allowed us to quantitate the ability of the cell to respond with a burst of superoxide production (17). We found that the ability of preincubated cells to respond to chemoattractants at 37°C appears to be quantitatively related to the calculated number of receptor sites remaining in the low affinity state and uncomplexed with the cytoskeleton. This desensitization was at least partially specific for formyl peptides (homologous) since other stimuli, such as C5a and phorbol myristate acetate, were partially or completely (respectively) effective in eliciting a response. Moreover, the desensitization and the formation of the ligand-receptor-cytoskeleton complex occurred more slowly at 4°C or in the presence of dihydrocytochalasin B (17, 18). Thus we proposed that this high affinity, Triton X-100–insoluble form of the receptor may be a form involved in the termination of the response of neutrophils to chemoattractants (17, 18).

Several questions remain unanswered in these studies. These questions concerned (*a*) the surface localization of the high affinity ligand-receptor complex, (*b*) the receptor nature of these binding sites, and (*c*) the molecular basis of the relationship between the progressive inability of the cell to respond and the association of ligand–receptor complex with the cytoskeleton in the high affinity form. In the study described below we endeavored to determine that the ligand-receptor complex observed was indeed a complex involving bona fide receptor as previously identified (22, 27) that was still at the cell surface after formation of the putative cytoskeletal association. In so doing, we discovered that after

neutrophil disruption by nitrogen cavitation, the plasma membrane separates into distinct fractions that can be resolved by sucrose density gradient sedimentation. This result suggested preservation of an interesting lateral order in the plane of the membrane. Since receptors in desensitized cells were observed to shift from one domain to another, this result also implied a segregation of receptors from their guanyl nucleotide-binding protein transduction partners in the plasma membrane that accompanied desensitization of the cell. Preliminary reports of these results have been published (2, 12).

Materials and Methods

Preparation of Cells

Human granulocytes were prepared from peripheral blood from healthy female donors by a modification (33) of an elutriation procedure originally described by Berkow et al. (3). The cells were treated with 2.5 mM diisopropylfluorophosphate for 15 min at 4°C, washed, and resuspended in Dulbecco's phosphate-buffered saline (DPBS)[1] consisting of 8 mM Na_2HPO_4 + 1.5 mM KH_2PO_4 + 2.7 mM KCl + 136.9 mM NaCl + 0.9 mM $CaCl_2$ + 0.5 mM $MgCl_2$, pH 7.4, containing superoxide dismutase (50 U/ml) and catalase (100 U/ml).

Surface Digestion of N-formyl Peptide Receptor and Preincubations with Ligand

Cells were divided into three aliquots (10^7 cells/ml) that were allowed to bind 5 nM radioiodinated, photoaffinity derivative of the chemotactic tetrapeptide, *N*-formyl-Met-Leu-Phe-N^{ε}-(2-(ρ-azido[^{125}I]salicylamido)ethyl-1,3'-dithiopropionyl)-Lys, or FMLPL-SASD-[^{125}I] (1) for 5 min at 4°C, 20 min at 15°C or 10 min at 37°C to produce majority populations of surface reversibly bound ligand, surface irreversibly bound ligand, and internalized ligand, respectively. The different incubation times did not affect any of the measured parameters except receptor ligand complex distribution (see also Results section). Subsequent to the incubation the ligand was covalently attached to its receptor by photolysis on ice for 10 min. The cells were then washed and resuspended in DPBS at 10^7 c/ml, and treated for 15 min on ice with either papain (500 μg/ml) in DPBS + 0.1 mM EDTA + 10 mM DTT + 60 μM β-mercaptoethanol that had been preactivated at 37°C for 10 min, or the same mixture that had been inactivated with 5 mM iodoacetamide (30 min/20°C). The cells were then washed twice in DPBS + 50 μg/ml chymostatin, 0.23 U/ml Trasylol, 2 mM PMSF and 5 mM iodoacetamide and solubilized by mixing 1:1 with 125 mM Tris, pH 6.8, + 6.7 M Urea + 12.5% β-mercaptoethanol + 2% SDS + 1 mM EDTA + 0.01% Bromophenol blue + chymostatin (50 μg/ml) + aprotinin (0.23 TIU/ml) + PMSF (2 mM) + leupeptin (5 mM).

Subcellular Fractionation of FMLPL-SASD[^{125}I]-labeled Granulocytes

After covalent photoaffinity labeling, the cells were diluted into 5 vol of ice cold DPBS, washed, and resuspended into sucrose-containing buffer in preparation for N_2 cavitation and subcellular fractionation by isopycnic sucrose density gradient sedimentation (14, 26). Subcellular fractions were assayed for myeloperoxidase, lactoferrin, and alkaline phosphatase as described by Parkos et al. (26). Determination of guanyl nucleotide-binding activity was performed as described by Northrup et al. (23). Detection of clathrin heavy chain was performed by ELISA in analogous fashion to the assay for lactoferrin using a 1:100 dilution of commercially available mouse monoclonal (IgG) anti-clathrin heavy chain (CHC 5.9 ICN Immunobiologicals, Lisle, IL) and peroxidase-conjugated goat anti-mouse IgG secondary antibody (Tago Labs, Inc., Burlingame, CA) at 1:1,000 dilution. Sucrose densities are given in weight percent ± SD.

1. *Abbreviations used in this paper*: FMLPL-SASD[^{125}I]; *F*-Met-Leu-Phe-N^{ε}-(2-(ρ-azido[^{125}I]salicylamido)ethyl-1,3'-dithiopropionyl)-Lys; DPBS; Dulbecco's phosphate-buffered saline; PM-L; low density plasma membrane; PM-H; high density plasma membrane.

SDS-PAGE

Electrophoresis was performed on a Laemmli type slab gel system (20, 25) with a number of important modifications. The stacking and separating gels were made in 0.1% SDS with 4 and 9% acrylamide respectively. They were 1.5-mm thick, 10-cm wide and 0.5- and 5-cm deep. The samples were diluted with an equal volume of solubilizing buffer containing 6.7 M urea, 175 mM Tris, 12.5% β-mercaptoethanol, 2% SDS, 0.01% Bromophenol Blue, and 1 mM EDTA and placed directly into the wells of the stacking gel. Samples were focused at a constant current of 20 mA and separated at a current of 40 mA. The gels were fixed and stained in 25% 2-propanol/10% acetic acid/0.05% Coomassie Brilliant Blue (G250) and destained in the same solvent without the dye.

Autoradiography

Gels were dried onto Whatman 3-MM Chr paper under vacuum and subsequently exposed to X-OMAT RP film (Eastman Kodak Co., Rochester, NY) with a Cronex Lighting Plus intensify screen (Dupont Instrument, Wilmington, DE). The film was developed after 2–5 d using a Konica QX-130A Processor (Konishinoko Photo Ind. Co. Ltd., Japan).

Western Blotting for Identification of Actin, Fodrin, Clathrin, and the Alpha and the Beta Chains of Guanyl Nucleotide Regulatory Proteins

Electrophoretic transfer of proteins from SDS-polyacrylamide slab gels onto nitrocellulose was performed according to Towbin et al. (34). After transfer the nitrocellulose strips (Millipore HA 0.45 μm, Millipore Corp., Bedford, MA) were soaked in saturating buffer consisting of 10% goat serum and 3% BSA (Sigma Chemical Co., St. Louis, MO) in 0.5 M NaCl and 10 mM Hepes pH 7.4. The nitrocellulose strips were then incubated overnight at 4°C with primary rabbit (or mouse) IgG in DPBS plus 3% goat serum, 1% BSA, and 0.2% Tween 20 (Sigma Chemical Co.). After rinsing the nitrocellulose five times with wash buffer consisting of 0.25 M NaCl, 10 mM Hepes, 0.2% Tween 20, pH 7.4, the strips were then incubated for 1 h at 20°C with 1 μg/ml of peroxidase-conjugated goat anti-rabbit (or mouse) IgG in DPBS plus 3% goat serum, 1% BSA and 0.2% Tween 20. Again after rinsing five times with wash buffer, the nitrocellulose strips were color developed for 5–30 min in a solution of developer consisting of 30% methanol, 0.5 mg/ml Bio-Rad peroxidase color developer (4-chloro-1-napthol), and 5 mM H_2O_2 in 0.25 M NaCl, 10 mM Hepes, pH 7.4. The reaction was terminated by the transfer of nitrocellulose strips to distilled water.

Affinity purified anti-actin antibody (IgG) was a kind gift of Dr. Keigi Fujiwara (7) and was used at a concentration of 1 μg/ml to detect actin in these blots. Normal rabbit serum IgG tested negative on transfers of neutrophil fractions and rabbit skeletal muscle actin. To detect the presence of G proteins in the fractions, antibodies (IgG) made against bovine brain beta-subunit (5) and against the carboxyl terminal nonapeptide (NNLKDCGLF) from the sequence of the alpha-subunit of G_i (4) were used at a concentration of 10 and 20 μg/ml respectively in the primary antibody incubation step. Rabbit antiserum raised against human brain fodrin (11) was the kind gift of Dr. Jon Morrow and was used at a dilution of 1:1,000 to detect the presence of fodrin. Mouse monoclonal (IgG anti-clathrin heavy chain) (CHC 5.9 ICN Immunobiologicals, Lisle, IL) and peroxidase-conjugated goat anti-mouse IgG secondary antibody (Tago Labs, Inc., Burlingame, CA) at 1:1,000 dilution were used to detect clathrin heavy chain. Unless explicitly stated all other materials were obtained from the sources quoted in the original references.

Results

Using both fluoresceinated and tritiated derivatives of chemotactic peptides, we have previously shown that after an incubation of 4°C, human neutrophils bind the chemoattractants reversibly ($t_{½}$ off <2 min) (16, 30) and are capable of responding to their continued presence after rapid warming of the cells to physiological temperatures. After identical incubations but at 15°C, most of the occupied ligand-binding sites are converted to a high affinity, virtually nondissociable ($t_{½}$ off >2 h) form (16, 28, 30) and are incapable of inducing

a response after warming (17, 28) in spite of their apparent surface localization (30). During incubations performed at 37°C the occupied receptors are internalized to a galactosyl transferase-enriched intracellular compartment (13, 15). We used the 15°C incubation as a way of trapping the receptor before endocytosis in a state allowing the analysis of the molecular basis for this affinity conversion in the plasma membrane.

To demonstrate that the converted binding sites were of surface receptor origin, we specifically labeled *N*-formyl peptide receptor on human neutrophils with a stimulatory dose of a radioiodinated, photoaffinity derivative of the chemotactic tetrapeptide, FMLPL-SASD[^{125}I] (1). Using this probe, we were able to compare the molecular weights and extracellular protease sensitivity of the cellular receptors. This comparison was done by covalently labeling the receptors while still on the cell with FMLPL-SASD[^{125}I] after the three temperature incubations described above, then treating the cells with papain (at 4°C) and solubilizing them in SDS. The receptors could then be identified by their characteristic mobility on SDS-PAGE as observed by autoradiography. The left panel of Fig. 1 shows that after incubation of the cells under the three conditions, only a single characteristic receptor species of M_r ~50–70 kD was labeled. The relative exposure of the film in the three lanes corresponding to the three preincubations, confirmed the previously reported relative amounts of radioligand uptake (nondissociable binding) after similar incubations. The 37°C preincubation resulted in the highest exposure and reflected continuous internalization and accumulation of the ligand-receptor complexes to the light Golgi (LG)-like (galactosyl transferase-enriched) fraction (13, 15). The 15°C preincubation was intermediate in exposure and the 4°C preincubation was the lowest. The difference in the magnitude of the labeling under these two latter conditions probably reflects different efficiencies of covalent incorporation arising from the differences in receptor affinities and the progressive decline in concentration of free photoactive ligand during irradiation of the cells. We conclude, therefore, that the converted forms of ligand binding observed under the three conditions resulted from modulation of the receptors and were not the result of the artifactual appearance of nonreceptor formyl peptide-binding sites.

To confirm the surface localization of receptor under the two low temperature conditions, we determined the protease sensitivity of the covalently labeled receptor in intact cells by its subsequent mobility on SDS-PAGE by autoradiography for the three conditions. The right panel of Fig. 1 shows that only the receptors in cells preincubated at lowered temperatures were completely sensitive to proteolysis, losing greater than 95% of the 50–70-kD species (by densitometry) and yielding the previously described 35-kD receptor fragment (9). Since the 37°C preincubation condition produces internalized ligand-receptor complex (15), which is 55% undigested, the experiment suggests that the complexes are indeed inaccessible to papain. The higher levels of low molecular weight degradation fragments, seen after the 37°C incubation, probably results from internal and endogenous degradation of ligand-receptor complexes and is present whether or not the cells are treated with papain. These fragments are not present after the two low temperature incubations, further supporting the surface localization of the labeled receptor in these cells. Comparison to controls not treated with papain or treated with inactivated papain also suggested no proteolysis occurred within the cell as there was no detectable difference in the Coomassie-staining patterns of the major protein constituents of the cell, nor in the intact morphology as observed by phase contrast microscopy (not shown).

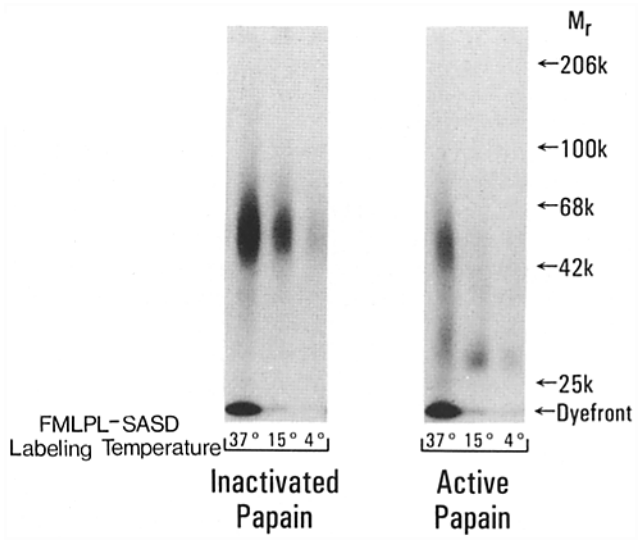

Figure 1. Surface localization of occupied *N*-formyl peptide receptors in ligand-desensitized human neutrophils. Neutrophils were divided into three aliquots (10^7 cells/ml) that were photoaffinity labeled with 5 nM FMLPL-SASD[^{125}I]for 5 min at 4°C, 20 min at 15°C or 10 min at 37°C, before being covalently coupled to receptor. The cells were then washed and resuspended in DPBS at 10^7 cells/ml, and treated with either activated papain (500 μg/ml) or iodoacetamide-inactivated papain. The cells were washed and solubilized in SDS-PAGE-solubilizing buffer containing a mixture of protease inhibitors. The samples were then subjected to SDS-PAGE and dried for autoradiography. In the autoradiogram of such a gel, the *N*-formyl peptide receptor (M_r = 50,000–70,000) was unaffected by inactivated papain. However, activated papain was able to cleave the receptor completely to produce a radiolabeled fragment of M_r ~35,000 in cells labeled at both 4°C and 15°C, but only partially in cells labeled at 37°C.

The sensitivity of the receptors to papain in the desensitized (15°C) and responsive cells (4°C) is strongly suggestive of their surface localization. In other studies using electron microscopic autoradiography to localize the ligand receptor complex, we also found that the two low temperature incubations resulted in equivalent grain distributions with 80–90% of the grains lying within 0.1 μm of the cell periphery (Jesaitis, A. J., and C. M. Chang, unpublished results). These results supported the above protease sensitivity and the nearly complete accessibility of fluorescent ligand to rapid (<1 s) quenching by low pH exposure after similar low temperature incubations (31).

To study the molecular interactions governing the desensitization process and to minimize the contamination of preparations with cytoplasmic cytoskeletal proteins irrelevant to high affinity complex formation, we performed a subcellular fractionation analysis of responsive and desensitized cells. Surprisingly, the results suggested that receptors were translocated to a novel plasma membrane subcompartment during the 15°C incubation. In these experiments, we prepared subcellular fractions from desensitized cells having

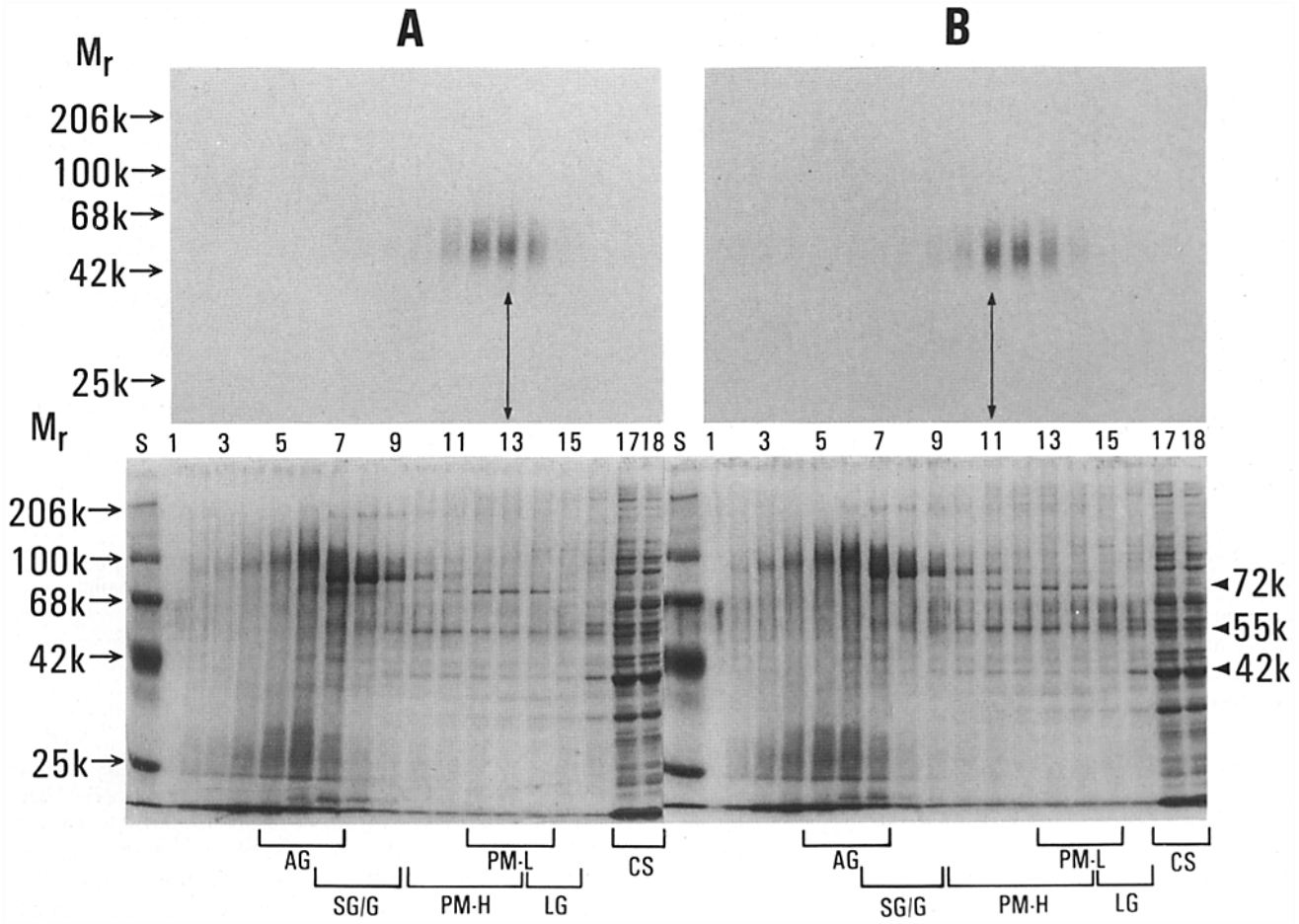

Figure 2. Transfer of occupied *N*-formyl peptide receptors from a light to a heavy plasma membrane domain during ligand-induced desensitization of human neutrophils. 6×10^8 neutrophils were resuspended in 4 ml DPBS and divided into two aliquots. The two-cell aliquots were photoaffinity labeled with 5 nM FMLPL-SASD[^{125}I], one at 4°C for 5 min and the other at 15°C for 15 min, followed by photolysis on ice for 10 min to covalently incorporate the ligand. The cells were then washed in DPBS, disrupted and fractionated as previously described (26). Fractions of 1.4 ml were collected and subjected to SDS-PAGE on 9% gels. The gels were fixed and stained with Coomassie Brilliant Blue (G-250), and dried down for autoradiography. The Coomassie Blue-staining profiles in the dried gels are shown in the lower panels. The upper panels show the autoradiograms of the same gels. The panels under *A* and *B* are of membrane fractions obtained from cells preincubated with FMLPL-SASD[^{125}I] at 4°C and 15°C, respectively. The fractions containing the highest activity of neutrophil subcellular organelles are indicated below the gel profiles: Azurophil granules (*AG*), Specific granules/Golgi (*SG/G*), light Golgi or endosomes (*LG*), cytosol (*CS*). The alignment of the peak in labeled receptor with the corresponding fraction of the stained gel is indicated by the arrow. In *A* alkaline phosphatase activity coincides with the receptor peak (lane *13*) and the peak staining density for the 72-kD band of the stained gels. This group of fractions has been termed the light plasma membrane (*PM-L*). However, the receptor in *B* (15°C cells) is shifted to a higher density (lane *11*), where it cosediments with proteins of M_r 55,000 and 42,000. This fraction is termed the heavy plasma membrane (*PM-H*). Specific granules can also be identified by lactoferrin at 78 kD.

the high affinity form of the receptor (incubated with FMLPL-SASD[^{125}I] at 15°C) and responsive cells having the low affinity form, (incubated with FMLP-SASD[^{125}I] at 4°C). The fractions were prepared by nitrogen cavitation of both cell populations after UV irradiation to covalently couple the ligand to the receptor. The cavitated homogenates were separated on isopycnic sucrose density gradients as previously described (26). Marker profiles were typically distributed with high recovery (>80%) in both gradients reproducing several previous studies from our laboratory (13, 15, 26). Aliquots were taken from the gradient fractions, run on SDS-PAGE and the resulting gels dried and used for autoradiography. Fig. 2 shows the resulting autoradiograms (upper panels) and the corresponding Coomassie Blue protein-staining patterns in the dried gels (lower panels) used for the autoradiography. The autoradiograms show that the characteristic receptor band of 50–70 kD is the only labeled species observed in any of the gradient fractions. It also shows that it is localized to the central fractions of the gradient which are enriched in plasma membrane markers but clearly separated from the heavy Golgi (galactosyl transferase) and granule (lactoferrin, myeloperoxidase) fractions in lanes *3–9* and the lighter, galactosyl transferase-enriched, endosomes or Golgi fractions in lanes *14–16.*

Closer inspection however, shows important and reproducible differences in the distribution of the photoaffinity-labeled ligand-receptor complexes isolated in these gradients. The peak exposure is observed in lane *13* in the fractions from responsive cells (*A*) corresponding to a sucrose density of 31.9 ± 0.8 weight percent ($n = 8$; $\rho = 1.135$ g/cc). It is, however, shifted to lane *11* (35.3 ± 1.7%; $n = 8$; $\rho =$ 1.155 g/cc) in fractions from desensitized cells exhibiting

conversion of receptors to the high affinity form. In both gradients the alkaline phosphatase activity, a common plasma membrane marker used in neutrophil studies sediments to the same sucrose density (30.7 ± 1.1% and 30.3 ± 0.8%, for the 4° and 15°C studies, respectively) with a peak corresponding to lanes *13* in Fig. 2, *A* and *B*. In *A* and *B* of Fig. 3 the relative distributions of alkaline phosphatase activity and receptor, quantitated by scanning densitometry, are shown for another identically run experiment which clearly shows the shift of receptor relative to the alkaline phosphatase activity. This latter invariant distribution was shown to accurately parallel the distribution of surface-binding sites for unliganded receptor, radioiodinated wheat germ agglutinin, surface radioiodinatable proteins, and cholesterol (26) (Jesaitis, A. J., J. O. Tolley, P. A. Hyslop, and R. A. Allen, unpublished results). The distribution of these markers also parallels a discrete set of Coomassie-stained bands observed in the lower panels. These include a major unidentified species of M_r = 72,000. Inspection of the fractions containing the shifted receptor, reveals that the protein composition of these fractions is distinct, containing a number of discrete bands including major species with M_r of 42,000 and 55,000, and that they are well resolved from the flanking granule and "plasma membrane fractions".

We have reproduced the shift in receptor localization in more than 16 similar experiments using cells isolated from different blood donors. In paired comparisons this shift was 3.8 ± 1.8 weight percent (n = 16) or ~10% of the working volume of the sucrose gradient, amounting to a significant 2 ml or a two fraction shift. The shift also occurs if the covalent labeling is done before the incubation at 15°C, thus indicating that the covalently labeled receptor species was not a new form to arrive at the cell surface replacing receptors that had been internalized and degraded at 15°C. We also observed the shift with use of the tritiated form of *F*-Met-Leu-Phe (2, 12) in which the conversion to high affinity form was responsible for retention of the peptide in the membranes after cell washing, cavitation and isolation of the membrane fractions. In two experiments, a misalignment of the receptor distribution with the 42- or 55-kD bands was observed which may have resulted from variabilities in the state of the cells obtained from the different blood donors. In addition, we found in other unpublished studies, that incubations at 4°C do not block, but only lower the rates of translocation to the heavy fraction or conversion to high affinity form of the receptor or desensitization of the cell. Furthermore, the differential in these rate (4° vs. 15°C) is also reduced when very high affinity ligands such as FMLPL-SASD (K_d ~ 0.3 nM) are used. Thus to observe the maximum differential translocation of receptor with the photoaffinity probe used here, we incubated the cells with FMLPL-SASD[^{125}I] for <5 min at 4°C and >15 min at 15°C. Controls were performed in which the times of incubation at 4°C were increased to match those at 15°C but these often resulted in partial translocation of receptor.

The resolved membrane fractions described here are also observed in fractions obtained from cells that have not been exposed to formyl peptides (not shown). Thus we presume they are preexisting in the cell and not due to changes induced by exposure of the cells to formyl peptides. Moreover, in other studies, we have also shown that a fraction of equivalent density, and shifted from the alkaline phosphatase distribution can be isolated from phorbol myristate acetate-

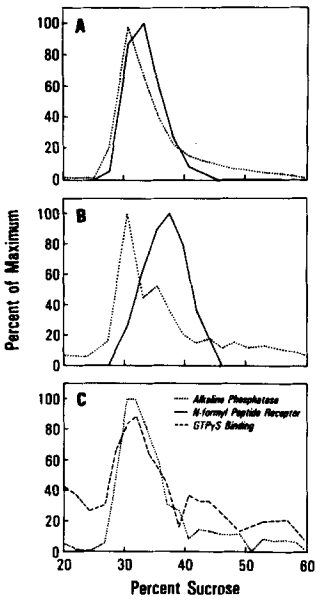

Figure 3. Partition of occupied *N*-formyl peptide receptors into a plasma membrane domain depleted of G-protein in ligand-desensitized human neutrophils. Neutrophils were DFP-treated, photoaffinity labeled, cavitated and fractionated by isopycnic sucrose density gradient sedimentation as described in Fig. 2. The profiles shown are: Alkaline phosphatase (·····), *N*-formyl peptide receptor (——), and GTPγ [^{35}S] binding (- - - -). *A* shows the cosedimentation of receptor with the plasma membrane marker alkaline phosphatase in responsive cells. *B* shows how the receptor localizes to a different domain when desensitized cells are fractionated. The position of alkaline phosphatase is unchanged. *C* shows that the GTPγ [^{35}S]-binding activity cosediments with alkaline phosphatase activity in desensitized cells (15°C) in contrast to the receptor.

stimulated neutrophils containing surface-localized, NADPH-dependent superoxide generating activity (Quinn, M. T., C. A. Parkos, and A. J. Jesaitis, unpublished results). Thus from cells treated entirely differently we observe similar separations thus suggesting a constancy in structure of the membrane at this level of resolution. Finally, because the occupied receptors in cells preincubated with the formyl peptide at 15°C were still accessible to exterior proteases, external acidification (31), and appeared by electron microscopic examination to be surface associated, receptor localization to this fraction suggests that this heavier membrane fraction is also surface derived and thus may represent a plasma membrane "microdomain". We therefore termed the two membrane fractions PM-L for the light plasma membrane fraction and PM-H for the heavy plasma membrane fraction.

Occupied *N*-formyl peptide receptors of neutrophils coisolate with a Triton X-100–insoluble fraction under the conditions which promote the shift of receptors to membrane fractions of higher density (17). Thus we wanted to determine whether there was a structural link between these events in the membrane. Since the PM-H fractions were the predominant locus of a 42-kD protein, we also examined the composition of the sucrose density gradient–isolated membrane fractions with respect to the presence of the cytoskeletal proteins actin, fodrin, and clathrin. Sucrose gradient fractions were separated by SDS–PAGE and transferred to nitrocellulose as described in the Materials and Methods section and blotted with antibodies specific to these proteins. After drying, these same blots were used to generate autoradiograms to monitor transferred photoaffinity-labeled receptor. Fig. 4 *A* is a composite overlay of such an autoradiogram and its corresponding Western blot. The gradient fractions shown were obtained from photoaffinity-labeled desensitized cells (15°C preincubation) and the immunoblots used affinity purified anti–actin antibodies. The figure shows that actin codistributes with receptor in these fractions and thus is present in the 42-kD band in evidence in the staining pattern.

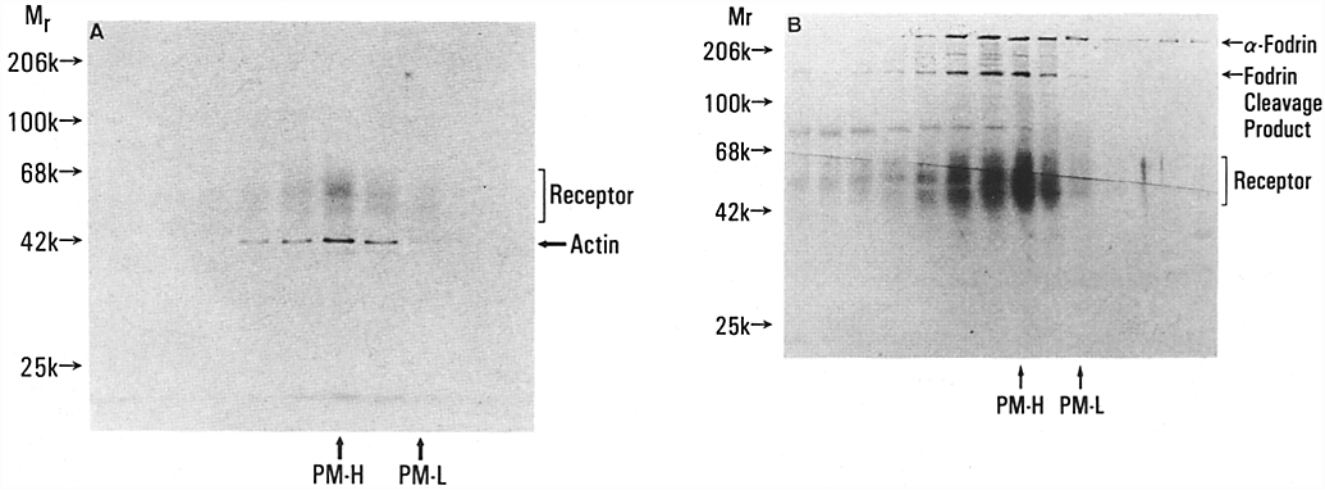

Figure 4. (*A*) Cosedimentation of actin with *N*-formyl peptide receptor in the PM-H domain of desensitized neutrophils. Fractions prepared as in Fig. 2 from formyl-peptide desensitized cells were analyzed for actin content. 300 μl of gradient fractions were diluted 1:1 with 10 mM Hepes, pH 7.4, and pelleted after 45 min at 45,000 rpm in a Beckman 50 Ti rotor at 4°C. Pellets were solubilized and subjected to SDS–PAGE on 9% gels as described in Fig. 1. The slab gels were electrophorectically transferred for Western blots to nitrocellulose (34) and bound with primary rabbit antibodies followed by peroxidase-conjugated goat anti-rabbit IgG as described (25) previously. Incubation with primary antibody (1 μg/ml) affinity purified rabbit anti-fish skeletal muscle actin (7) was performed overnight at 4°C in blocking buffer (25). (*B*) Cosedimentation of the alpha subunit of fodrin (M_r = 240,000) with the shifted *N*-formyl peptide receptor in the heavy plasma membrane fractions (*PM-H*) obtained in cells incubated with FMLPL–SASD[^{125}I] at 15°C. These experiments were performed and are displayed as described in *A* with the exception that the shifted receptors were covalently labeled before their incubation at 15°C. A 1:1,000 dilution of anti-human fodrin antisera was used as the primary antibody. The lighter band of M_r ~150,000 is the proteolytic fragment of alpha-fodrin resulting from activity of the calcium-sensitive protese (11).

In the corresponding fractions from responsive cells (4°C preincubation) the receptor is in the PM-L and shifted relative to the invariant actin profile (not shown). Fig. 4 *B* shows that the receptor also cosediments with alpha-fodrin and its 150-kD, Ca^{++}-sensitive cleavage product.

Attempts to detect clathrin heavy chain using commercially available anti–clathrin antibodies gave consistent detection of clathrin in the cytosolic fractions, but inconsistent results for the membrane fractions. Micro-ELISA assays supported the detection of clathrin in the cytosolic fraction and the dense granule fraction but not in the membrane fractions. Because of the low levels of coated pits observed in these cells and the apparent lack of correlation of these structures with the autoradiographic grains of iodinated *N*-formyl peptide observed during statistical analysis of grain distribution by electron microscopy (16) the relationship of these structures to the formation of the converted high affinity surface-receptor sites observed after the 15°C incubation remains unresolved.

To determine whether the ligand-induced desensitization and receptor transfer had a possible functional relationship, we analyzed the subcellular distribution of guanyl nucleotide regulatory proteins in the sucrose density gradient fractions of the responsive (4°C) and densensitized (15°C) cells. Fig. 3 shows that Gamma[^{35}S]GTP-binding activity (*C*) co-sedimented with the plasma membrane marker activity, alkaline phosphatase, in fractions derived from FMLPL–SASD-[^{125}I]-desensitized and responsive cells but not with ligand–receptor complexes in the desensitized cells (Fig. 3, *A* and *B*). This distribution was confirmed using a rabbit polyclonal antibody recognizing the pertussis toxin substrate, the alpha subunit of human neutrophil G-protein and is shown in Fig. 5. A parallel distribution was also observed for the beta-subunit (not shown). Based on the GTP-binding measurements and immunoblots, we estimate that the vesicles (PM-H) containing shifted receptor have a significantly lower G-protein content (10–20%) than the alkaline phosphatase-enriched, PM-L vesicles. This relative depletion suggests a different environment for the receptors in the two fractions.

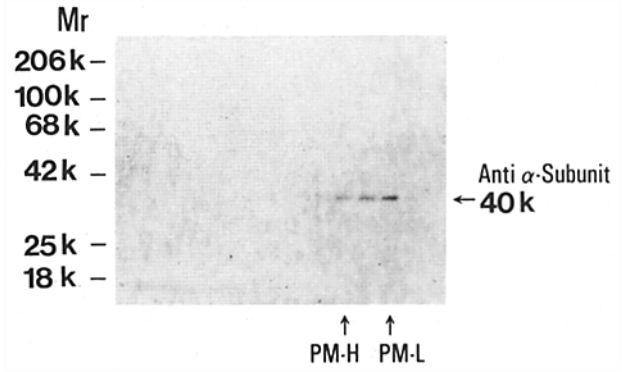

Figure 5. Cosedimentation of the alpha subunit of the guanyl nucleotide regulatory protein with the PM-L domain (fodrin and actin-depleted) of formyl peptide desensitized human neutrophils. Fractions used in the experiment shown in Fig. 3 were also analyzed for G-protein content using an anti–alpha chain antibody. Peroxidase activity of the conjugated secondary antibody was observed to localize in the fractions enriched in PM-L domain (*PM-L*) and clearly resolved from the receptor and actin shown in Fig. 4 in the heavy plasma membrane domain (*PM-H*).

Discussion

The purpose of the investigation described in this paper was to explore the molecular basis for regulation of chemotactic receptors of human neutrophils at the plasma membrane level. Our particular emphasis was to investigate the role of the cytoskeleton in receptor modulation in the plasma membrane. We hoped to relate this function to the desensitization of human neutrophils to chemoattractants and conversion of these chemotactic receptors to a high affinity, very slowly dissociating and Triton X-100–insoluble form previously described (16, 17, 35). In this study we were able to show that the high affinity ligand-binding sites for *N*-formyl peptides observed after incubation of neutrophils with such peptides at 15°C were due to bona fide receptors identified by their M_r on SDS–PAGE and that these receptors were localized to the cell surface by several independent criteria. The most interesting result from our study suggests that these receptors are translocated from a plasma membrane domain enriched guanyl nucleotide regulatory protein to a domain depleted in these proteins but enriched in proteins of the membrane skeleton, actin, and fodrin.

The interpretation of these results relies on the assumption that the N_2 cavitation of human neutrophils in sucrose at low ionic strength fragments the plasma membrane into at least two vesicle populations of differential composition. The PM-L (light) fraction cosediments with a number of plasma membrane marker activities including alkaline phosphatase, surface iodinatable and surface lectin-labeling proteins, and G-proteins. The PM-H fraction is characterized as the major sedimentable actin and fodrin locus of the cell and in similar preparations the locus of a surface NADPH-dependent superoxide generating enzyme system (Quinn, M. T., C. A. Parkes, and A. J. Jesaitis, unpublished results). In addition, occupied *N*-formyl peptide receptors can be moved from PM-L to PM-H without significantly affecting their precavitation sensitivity to extracellular proteases, accessibility to fluorescence quenching by rapid extracellular acidification (30), or their localization by electron microscopy autoradiography. Although the likelihood is very small, without application of specific alternative probes (e.g., antibodies) which can clearly show the extracellular orientation of the receptor-binding sites, we cannot rule out localization of these receptors to a subsurface, vesicular pool which is rapidly accessible to papain or hydrogen ions at 4°C.

Reports of isolation of structural domains of plasma membrane in platelets (8) and lymphoid cells (10, 19) have been published as have reports of actin-rich domains associated with the phagocytic process (32). In neutrophils, the domain we describe appears to preexist in membrane and appears functionally related to control of surface chemotactic receptors. In addition our results suggest that all the latent alkaline phosphatase activity originating from intracellular pool reported by Borregaard et al., (6) has been expressed during isolation of the cells and has no apparent relationship to the fractions under study.

We have interpreted the translocation of occupied receptors from PM-L to PM-H as another manifestation of the conversion of receptors to the Triton X-100–insoluble, high affinity form found in preponderance in cells desensitized by prior incubation with ligand at 15°C. Their physiological relevance derives from our kinetic studies of these latter events at 37°C both in isolated membrane and isolated cytoskeletons. Recent work by Painter and associates (25) suggesting that this form is insensitive to guanyl nucleotides has now been confirmed by our own studies using membranes containing such high affinity receptors bound noncovalently with the tritiated form of F-Met-Leu-Phe. In these unpublished studies we showed that tritiated *F*-Met-Leu-[^{3}H]Phe, could not be induced to dissociate, by GTP-gamma-S from membrane obtained from cells preincubated with the radioligand (Cupo, J. F., and A. J. Jesaitis, unpublished results). Together these results suggest that the high affinity form of the occupied receptors found on the surface of cells at 15° and 37°C are incapable of interacting with the G-proteins. Our results, in contrast with those of Painter et al. (24) however, suggest that the demonstrated insensitivity of receptors to guanyl nucleotides might alternatively or additionally arise from a lateral segregation of receptors from their G-protein transducing partners. If such segregated receptors could be reconstituted in membrane vesicles containing G-proteins and demonstrate guanyl nucleotide sensitivity in their binding characteristics, then the lateral segregation observed could be invoked as a possible mechanism of control of chemoattractant activation of human neutrophils.

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