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\textsuperscript{N'-}(2-(p-azido\textsuperscript{[125I]}salicylamido)ethyl-1,3'-dithiodpropionyl)-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 (p \approx 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 (p \approx 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\textsubscript{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 cytoskeleton of the cell. In our studies, we suggested that the coisolation of cell surface ligand-receptor complexes with the Triton X-100-insoluble cytoskeleton 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 caviation, 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 the 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 di-isopropylfluorophosphate for 15 min at 4°C, washed, and resuspended in Dulbecco's phosphate-buffered saline (DPBS) consisting of 8.1 mM Na2HPO4 + 1.5 mM KH2PO4 + 2.7 mM KCl + 136.9 mM NaCl + 0.9 mM CaCl2 + 0.5 mM MgCl2, 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 (107 cells/ml) that were allowed to bind 5 nM radiolabeled, photaffinity derivative of the chemotactic tetrapeptide, N-formyl-Met-Leu-Phe-NH2-(2-(p-azido[125I]salicylamido)ethyl-1,3-dithiopropionyl)-Lys, or FMLP–SASD-[125I] (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 107 c/ml, and treated for 15 min on ice with either papain (500 mg/ml) in DPBS + 0.1 mM EDTA + 10 mM DTT + 60 μM β-mercaptoethanol that had been preincubated 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 + 1.5 mM KCl + 136.9 mM NaCl + 0.9 mM CaCl2 + 0.5 mM MgCl2, pH 7.4, containing superoxide dismutase (50 U/ml) and catalase (100 U/ml).

Subcellular Fractionation of FMLP–SASD-[125I]-labeled Granulocytes

After covalent photaffinity labeling, the cells were divided into 5 vol of ice cold DPBS, washed, and resuspended into sucrose-containing buffer in preparation for N2 caviation 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 Immunobiolog-icals, 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.

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 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 using a Konica QX-130A Processor (Konishindo 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 incubated 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-naphtol), and 5 mM H2O2 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. Keiji Fujisawa (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 neutrophils. Mouse monoclonal (IgG anti-clathrin heavy chain) (CHC 5.9 ICN Immunobiolog-icals, 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 (t50 <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 nons dissociable (t50 >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, FMLP–SASD\(^{[125I]}\) (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 FMLP–SASD\(^{[125I]}\) 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 \(M_r \sim 50-70\) 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).

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 \(\mu\)m 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 x 10^6 neutrophils were resuspended in 4 ml DPBS and divided into two aliquots. The two-cell aliquots were photoaffinity labeled with 5 nM FMLP-SASD[125I], 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 FMLP-SASD[125I] 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 Mr 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 FMLP-SASD[125I] at 15°C) and responsive cells having the low affinity form, (incubated with FMLP-SASD[125I] 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; ρ = 1.135 g/cc). It is, however, shifted to lane 11 (35.3 ± 1.7%; n = 8; ρ = 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, radiolabeled wheat germ agglutinin, surface radioligand, 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, = 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, 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 (Kd ~ 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[125I] 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-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 electrophoretically 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 (Mr = 240,000) with the shifted N-formyl peptide receptor in the heavy plasma membrane fractions (PM-H) obtained in cells incubated with FMLPL-SASD[125I] 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 Mr, ~150,000 is the proteolytic fragment of alpha-fodrin resulting from activity of the calcium-sensitive protease (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 desensitized (15°C) cells. Fig. 3 shows that Gamma[35S]GTP-binding activity (C) co-sedimented with the plasma membrane marker activity, alkaline phosphatase, in fractions derived from FMLPL-SASD[125I]-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 the cytoskeleton in receptor modulation in the plasma membrane. To explore the molecular basis for regulation of chemotactic receptors, we focused on the demonstration of receptors in the plasma membrane that are capable of interacting with the G-proteins. Our results suggest that the high affinity form of the occupied receptors found on the surface of cells at 15°C and 37°C are incapable of interacting with the G-proteins. We, 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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References

1. Allen, R. A., J. O. Tolley, and A. J. Jesaitis. 1986. Preparation and properties of an improved photoaffinity ligand for the N-formyl peptide receptor. Biochim. Biophys. Acta. 882:271–280.

2. Allen, R. A., A. E. Traynor, G. M. Omann, and A. J. Jesaitis. 1988. The chemotactic peptide receptor: a model for future understanding of chemotactic disorders. Hematology/Oncology Clinics of North America Vol. 2(1). Phagocytic defects I: Abnormalities outside of the respiratory burst. J. T. Curnutte, editor. W. B. Saunders Co., Philadelphia, Pennsylvania. 33–59.

3. Berkow, R. L., D. Y. Tzeng, L. V. Williams, and R. L. Baehner. 1983. The comparative responses of human polymorphonuclear leukocytes obtained by counter flow centrifugal elutriation and Ficoll-Hypaque density centrifugation. J. Lab. Clin. Med. 102:732–742.

4. Bokoch, G. B., K. Bickford, and B. P. Bohl. 1988. Subcellular localization and quantitation of the major neutrophil peroxisome substrate, Gm. J. Cell. Biol. In press.

5. Bokoch, G. M., L. A. Sklar, and J. E. Smolen. 1987. Guanine nucleotide regulatory proteins as transducers of receptor-stimulation neutrophil activation. Int. J. Tiss. 9:285–293.

6. Borregaard, N., L. J. Miller, and T. A. Springer. 1987. Chemoattractant-regulated mobilization of a novel intracellular compartment in human neutrophils. Science (Wash. DC). 237:1204–1205.

7. Borregaard, N., T. Christiansen, H. R., and K. Fujikawa. 1986. Stress fibers in cells in situ: Immunofluorescence visualization with antiaetin, antiamyosin, and antialpha actinin. J. Cell. Biol. 93:804–811.

8. Crawford, N. 1985. Structure and molecular properties of platelet membrane systems. In Platelet Membrane Glycoproteins. J. N. George, A. J. Nurden, and D. R. Phillips editor. Plenum Press, New York and London.

9. Dolmatch, B., and J. Niedel. 1983. Formyl peptide chemotactic receptor. Evidence for an active proteolytic fragment. J. Biol. Chem. 258:7570–7577.

10. Goppelt, M., R. Eichhorn, G. Krebs, and K. Resch. 1986. Lipid composi...
11. Harris, A. S., L. A. Green, K. J. Ainger, and J. S. Morrow. 1985. Mechanism of cytoskeletal regulation (I): functional differences correlate with antigenic dissimilarity in human brain and erythrocyte spectrin. Biochem. Biophys. Acta. 854:184-190.

12. Jesaitis, A. J., G. M. Bokoch, and R. A. Allen. 1988. Regulation of signal transduction in human neutrophils by cytoskeleton-mediated reorganization of the plasma membrane chemotactic receptor. In Signal Transduction in Cytoplasmic Organization and Cell Motility. In press.

13. Jesaitis, A. J., J. R. Naemura, R. G. Painter, M. Schmitt, L. A. Sklar, and C. G. Cochrane. 1985. The fate of the N-formyl-chemotactic peptide receptor in stimulated human granulocytes: subcellular fractionation studies. J. Cell. Biochem. 20:177-191.

14. Jesaitis, A. J., J. R. Naemura, R. G. Painter, L. A. Sklar, and C. G. Cochrane. 1982. Intracellular localization of N-formyl chemotactic receptor and Mg++ dependent ATPase in human granulocytes. Biochem. Biophys. Acta. 719:556-568.

15. Jesaitis, A. J., J. R. Naemura, R. G. Painter, L. A. Sklar, and C. G. Cochrane. 1983. The fate of an N-formylated chemotactic peptide in stimulated human granulocytes: subcellular fractionation studies. J. Biol. Chem. 258:1968-1977.

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

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

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

19. Klausner, R. D., A. M. Kleinfeld, R. L. Hoover, and M. J. Karnovsky. 1980. Lipid domains in membranes: evidence derived from structural perturbations induced by free fatty acids and lifetime heterogeneity analysis. J. Biol. Chem. 255:1286-1295.

20. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (Lond.). 227:680-685.

21. Manara, F. S., and D. L. Schneider. 1985. The activation of the human neutrophil respiratory burst occurs only at temperatures above 17 degrees C: evidence that activation requires membrane fusion. Biochem. Biophys. Res. Commun. 10:696-701.

22. Niedel, J., J. Davis, and P. Cuatrecasas. 1980. Covalent affinity labeling of formyl peptide chemotactic receptors. J. Biol. Chem. 255:7063-7066.

23. Northrup, K. M., D. Smigel, and A. G. Gilman. 1982. The guanylyl nucleotide activating site of the regulatory site of the regulatory component of adenyl cyclase. J. Biol. Chem. 257:11416-11423.

24. Painter, R. G., K. Zahler-Benzis, and R. E. Dukes. 1987. The regulation of the affinity state of the N-formylated peptide receptor of neutrophils: The role of guanyl nucleotide-binding proteins and the cytoskeleton. J. Cell. Biol. 105:2959-2972.

25. Parkos, C. A., R. A. Allen, C. G. Cochrane, and A. J. Jesaitis. 1987. Purified cytochrome b from human granulocyte plasma membrane is composed of two polypeptides with relative molecular weights of 91,000 and 22,000. J. Clin. Invest. 280:732-742.

26. Parkos, C. A., C. G. Cochrane, M. Schmitt, and A. J. Jesaitis. 1985. Regulation of the oxidative response of human granulocytes to chemotactants: no evidence for stimulated traffic of redox enzymes between endo and plasma membranes. J. Biol. Chem. 260:6541-6547.

27. Schmitt, M., R. G. Painter, A. J. Jesaitis, K. Preissner, L. A. Sklar, and C. G. Cochrane. 1983. Photoaffinity labeling of the N-formyl peptide receptor binding site of intact human polymorphonuclear leukocytes: a label suitable for following the fate of the receptor-ligand complex. J. Biol. Chem. 258:649-654.

28. Showell, H. J., D. Williams, E. L. Becker, P. H. Naccache, and R. Sha'afi. 1979. Densensitization and deactivation of the secretory responsiveness of rabbit neutrophils induced by the chemotactic peptide, Formylmethionyl-leucyl-phenylalanine. J. Receptor Res. 25:139-150.

29. Sklar, L. A., G. M. Bokoch, D. Button, and J. E. Smolen. 1987. Regulation of ligand-receptor dynamics by guanine nucleotides. Real-time analysis of interconverting states for the neutrophil formyl peptide receptor. J. Biol. Chem. 262:135-139.

30. Sklar, L. A., D. A. Finney, Z. G. Oades, A. J. Jesaitis, R. G. Painter, and C. G. Cochrane. 1984. The dynamics of ligand-receptor interactions: Real-time analysis of association, dissociation, and internalization of an N-formyl peptide and its receptors on the human neutrophil. J. Biol. Chem. 259:5661-5669.

31. Sklar, L. A., A. J. Jesaitis, R. G. Painter, and C. G. Cochrane. 1982. Ligand/receptor internalization: a spectroscopic analysis and comparison of ligand binding, cellular response, and internalization by human neutrophils. J. Cell. Biochem. 20:193-202.

32. Stewart, D. H., and N. Crawford. 1985. Redistribution of membrane-bound and cytosolic actin in rabbit polymorphonuclear leucocytes during phagocytosis. Biochem. J. 225:807-814.

33. Tolley, J. O., G. M. Omann, and A. J. Jesaitis. 1987. A high yield, high purity elutriation method for preparing human granulocytes demonstrating enhanced experimental lifetimes. J. Leuko. Biol. 42:43-50.

34. Towbin, H., T. Stachelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. 76:4350-4354.

35. Zagmound, S. H., and A. W. Tranquillo. 1986. Chemotactic peptide binding by rabbit polymorphonuclear leukocytes: Presence of two compartments having similar affinities but different kinetics. J. Biol. Chem. 261:5283-5288.