

Regulation of Chemoattractant Receptor Interaction with Transducing Proteins by Organizational Control in the Plasma Membrane of Human Neutrophils

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Abstract. Isolated purified plasma membrane domains from unstimulated human neutrophils were photoaffinity labeled with *F*-Met-Leu-Phe-*N*'-(2-(*p*-azido-[¹²⁵I]salicylamido)ethyl-1,3'-dithiopropionyl)-Lys also referred to as FMLPL-SASD[¹²⁵I]. Most of the photoaffinity-labeled *N*-formyl peptide receptors were found in light plasma membrane fraction (PM-L) which has been previously shown to be enriched in guanyl nucleotide binding proteins and the plasma membrane marker alkaline phosphatase (Jesaitis, A. J., G. M. Bokoch, J. O. Tolley, and R. A. Allen. 1988. *J. Cell Biol.* 107:921-928). Furthermore, the heavy plasma membrane fraction (PM-H), which is enriched in actin and fodrin, was depleted in receptors. Solubilization of PM-L and PM-H in divalent cation-free buffer containing octylglucoside and subsequent sedimentation at 180,000 *g* in detergent-containing sucrose gradients revealed two receptor forms. The major population, found in PM-L sedimented as a globular protein with an apparent sedimentation coefficient of 6-7S, while a minor fraction found in the PM-H fraction sedimented as a 4S particle. In addition, the 6-7S form could be converted to the 4S form by inclusion of guanosine 5'-*O*-(3-thiotriphosphate) (GTP γ S) in the extraction buffer (ED₅₀ = 10-30 nM). ATP was not effective at doses of up to 10 μ M. In contrast, iso-

lation and solubilization of receptors from desensitized cells (photoaffinity labeled after a 15°C incubation with FMLPL-SASD[¹²⁵I]) revealed that the majority of receptors (>60-90%), which are found in PM-H, sedimented as 4S particles. A minor fraction of receptors found in the PM-L sedimented as 6-7S species. The receptors in the PM-H fraction, however, were still capable of interacting with G-proteins, since addition of unlabeled PM-L membrane fraction as a G-protein source reconstituted a more rapidly sedimenting form showing sensitivity to GTP γ S. These results suggest that receptors in unstimulated human neutrophils have a higher probability of interacting with G-proteins because they are in the light plasma membrane domain. The results also suggest that receptors that have been translocated to the heavy plasma membrane domain during the process of desensitization or response termination have a lower probability of interacting with G-proteins. Since the latter receptors are still capable of forming G protein associations, then their lateral segregation would represent a mechanism of controlling of receptor G-protein interactions. This reorganization of the plasma membrane, therefore, may form the molecular basis for response termination or homologous desensitization in human neutrophils.

HUMAN neutrophils (polymorphonuclear leukocytes) form the body's front line defense against infection by microorganisms (10, 11, 13). They accomplish this essential function using a highly regulated sensory transduction system that can activate a variety of responses which include chemotaxis, secretion, adherence, and production of superoxide anion (8, 26). When the regulation of these functions is faulty the result can be disastrous for the host, producing serious chronic and acute inflammation or the inability to fight infection (13).

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Many of this cell's responses are triggered when specific receptors bind ligand and activate a transduction cascade via the occupied receptor's interaction with guanyl nucleotide-binding proteins. These G-proteins "transduce" the occupancy of receptors in a variety of systems including those mediating hormone, visual, mechanical, and chemosensory cell activation (12). One such system in the human neutrophil is that triggered by the occupancy of *N*-formyl chemotactic peptide receptors by simple *N*-formylated oligopeptides (4, 26). Occupancy of this receptor can trigger, under different circumstances, most of the neutrophil's responses (4, 8, 25).

Recent work in our laboratory (see reference 14 for summary) demonstrated that during a response at physiological

temperatures, surface receptors formed a transient high affinity, slowly dissociating form found in coisolation with the cytoskeleton of the cell. Cytochalasin B potentiated the response of neutrophils by both prolonging it and increasing its magnitude of the response, while inhibiting coisolation of the high affinity form with the cytoskeleton. Thus we speculated that the receptor-cytoskeletal association and affinity conversion may be involved with the termination of the response of the neutrophil to formyl peptides. Subsequently, we showed that a quantitative relationship existed between the number of receptors not converted to this high affinity form and the ability of the cell to respond to formyl peptides. This result provided support for our hypothesis that the conversion apparently interferes with the ability of these receptors to participate in transduction. We postulated that these receptors enter a state distinct from the higher affinity state found when receptors are coupled to G-proteins (14, 23). Indeed, when generated at 37°C the former class of receptors are still at the cell surface and can be isolated in plasma membranes or in association with the cytoskeleton in a form unable to dissociate in the presence of guanyl nucleotides (15, 19, 23). These results supported the hypothesis that the receptors were inactivated and uncoupled from G-proteins.

Our most recent work has confirmed that the high affinity receptors are indeed still in the plasma membrane. The alternate hypothesis, of their internalization to a plasma membrane-like endosomal compartment, has been ruled out by three independent criteria. These include: (a) accessibility of receptors to extracellular papain digestion (13); (b) surface localization by electron microscopic autoradiography (13); and (c) sensitivity of fluorescence of fluorescein-bound receptors to extracellular acidification (24). However, when homogenates of desensitized cells were subjected to fractionation by isopycnic sucrose density sedimentation, the surface receptors appeared to be reproducibly resolved from the conventional plasma membrane marker, alkaline phosphatase. The protein composition of this shifted fraction was significantly different, being enriched in actin and fodrin but depleted in guanyl nucleotide binding proteins. Hence it was concluded that this population of receptors in desensitized cells was localized to a separated plasma membrane domain (13). This work suggested that the receptors might be physically segregated from such proteins in the plane of the membrane, thus raising the possibility that the segregation might control response.

In this report we present physical evidence to support this hypothesis. We show that two different interconvertible hydrodynamic forms of N-formyl peptide receptor can be isolated in octyl glucoside extracts of the light and heavy plasma membrane microdomains of the human neutrophil. Solubilization of the light fraction produces a rapidly sedimenting species while solubilization of the heavy fraction results in a slowly sedimenting species. The rapidly sedimenting form predominates in cells that have had no earlier exposure to formyl peptide. The slowly sedimenting form predominates in cells that have earlier exposure to peptide under desensitizing conditions. The rapid form can also be converted to the slow form by exposure to guanyl nucleotides. The slow form can be converted back to the GTP-sensitive rapid form by solubilization with added G-protein-enriched membrane. We conclude, therefore, that receptors still can potentially associate with G-proteins after their conversion to the high

affinity form that accompanies the desensitization of the cells. However, they are prevented from doing so by their physical segregation from their transduction partners in the plane of the plasma membrane. This process, therefore, might serve as a regulatory mechanism of receptor-G-protein interaction.

Materials and Methods

Buffers, chemicals, methods of cell preparation, fractionation, photoaffinity labeling, and procedures for SDS-PAGE, autoradiography, and Western blot analysis were previously described by Jesaitis et al. (15). Guanosine 5'-O-(3-thiotriphosphate) (GTP γ S)¹ was purchased from Sigma Chemical Co. (St. Louis, MO).

Photoaffinity Labeling of Membrane Receptors

Photoaffinity labeling of isolated membranes was performed according to the procedure described by Allen et al. (3). After preparing neutrophils, plasma membrane fractions were prepared by isopycnic sucrose density gradient sedimentation. The relevant plasma membrane fractions were selected by measuring sucrose density, alkaline phosphatase activity, or receptor-specific radioactivity of photoaffinity-labeled N-formyl peptide receptors. Relevant fractions were used directly or pooled. Generally, fractions for pooling were chosen by sucrose percentage based on previous studies (15): 29–32% sucrose for light plasma membrane fractions (PM-L), 33–36% for heavy plasma membrane fractions (PM-H), or 30–35% for PM-L + PM-H. To any given volume of membrane pool (0.5–3.0 ml) an equal volume of 10 mM Hepes buffer, pH 7.4, was added followed by addition of ~1–5% of the volume of *F*-Met-Leu-Phe-*N*-(2-(p-azido[¹²⁵I]salicylamido)ethyl-1, 3'-dithiopropionyl)-Lys (FMLPL-SASD[¹²⁵I]) in 20 mM NaOH to bring the final concentration of the photoaffinity ligand to 5 nM. The mixture was incubated on ice for 15 min and covalently coupled by exposure to U.V. light in a Rayonet Photochemical Reactor (Southern New England Ultraviolet Co., Middlefield, CT) for 10 additional minutes while on ice. The membranes were then further diluted if necessary and sedimented in a rotor (model 50Ti; Beckman Instruments Inc., Palo Alto, CA) centrifuged at 45,000 rpm for 45 min. The supernatants were aspirated and discarded.

Solubilization of Photoaffinity-labeled Membrane Receptors

Methods for solubilizing membrane fractions and sedimenting the extracts were essentially the same as described by Parkos et al. (20) with the exception that the 1 M NaCl wash of the membranes or 100,000 *g* precentrifugation were not used. In brief, membrane pellets were resuspended in 300 μ l of buffer containing 100 mM KCl, 10 mM NaCl, 1 mM EDTA, 10 mM Hepes, 1% octylglucoside, pH 7.4, by agitation and allowed to extract for 1–2 h on ice. Membrane protein concentration of the extract was varied between 0.1 and 0.3 mg/ml.

Velocity Sedimentation of N-Formyl Peptide Receptors

Octylglucoside extracts (200 μ l) were layered directly onto 5 ml 5–20% linear sucrose density gradients made in the extraction buffer. Gradients were centrifuged in a swinging bucket rotor (model SW50.1; Beckman Instruments Inc.) for 16–17 h at 45,000 rpm. Sedimentation runs were calibrated with known protein standards by centrifuging a mixture containing 50 μ g each of cytochrome *c* (2.1S), BSA (4.4S), rabbit immunoglobulin (7.7S), and bovine catalase (11.2S) in parallel with the experimental gradients. 400- μ l fractions were collected and assayed for ¹²⁵I content with an ISO-DATA 20/20 series gamma radiation counter (Iso-Data, Rolling Meadows, IL). In addition, to identify receptor specific radioactivity and sedimentation rates of the standards, gradient fractions were diluted 1:1 in sample buffer for analysis by SDS-PAGE and/or autoradiography as described previously (3, 15). Procedures corresponding to each experiment are detailed in the figure legends.

1. *Abbreviations used in this paper:* GTP γ S, guanosine 5'-O-(3-thiotriphosphate); PM-L, light plasma membrane fractions; PM-H, heavy plasma membrane fractions.

Results

Isolation of Photoaffinity-labeled N-Formyl Peptide Receptors from Plasma Membrane of Human Neutrophils

To study the molecular associations of the N-formyl peptide receptors, alkaline phosphatase-enriched plasma membrane fractions were isolated from isopycnic sucrose density gradient fractions of N_2 cavities prepared from unstimulated cells. As described previously (15), these fractions are enriched in receptors and G-proteins. The receptors were labeled with the photoaffinity ligand N-formyl Met-Leu-Phe-Lys-SASD $^{[125I]}$. The labeled membranes were solubilized in a divalent cation-free buffer of neutral pH containing potassium and octylglucoside. This extract was then sedimented overnight for 17 h on a 5-ml sucrose density gradient (5–20% [wt/wt]) made up in the same buffer. The distribution of the

receptor in the fractionated gradient as visualized by SDS-PAGE autoradiography is shown in Fig. 1 A. The 50–70-kD photoaffinity-labeled receptor sedimented as a broad band species of apparent sedimentation coefficient of ~ 6 –7S. Such a sedimentation coefficient is characteristic of globular proteins of molecular mass almost twice that of the receptor as observed on SDS-PAGE. Moreover, since the measured partial specific volume of the detergent solubilized purified receptor is ~ 0.88 cm 3 /g (2), the result suggests that the receptor was probably part of an oligomeric complex with other proteins.

Guanyl Nucleotide Sensitivity of Receptor-bearing Sedimentable Complex

A logical candidate for forming a molecular complex with receptor in the plasma membrane would be a member of the class of transducing proteins or guanyl nucleotide binding

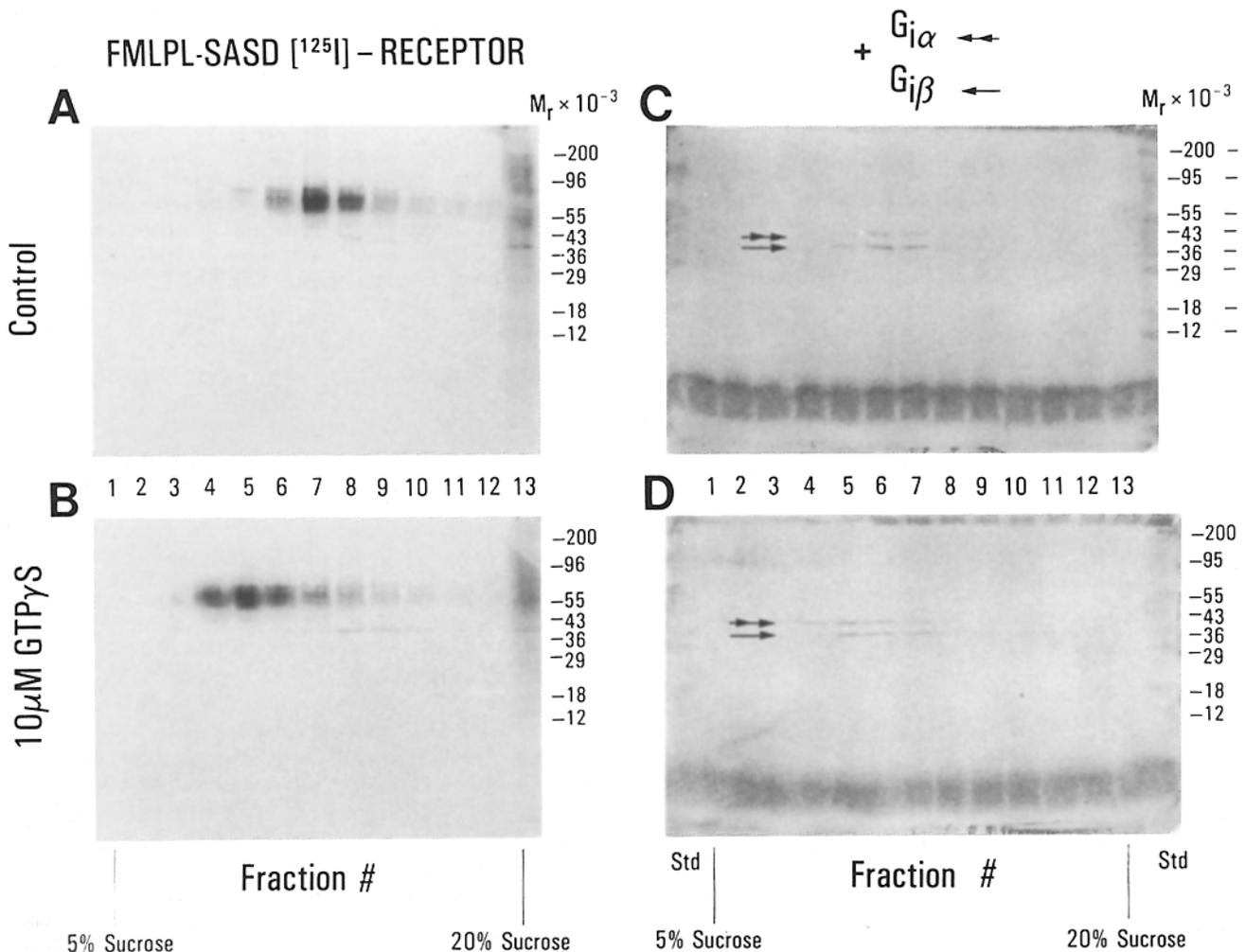


Figure 1. (A and B) Rate zonal ultracentrifugation of detergent extracts of plasma membranes obtained from unstimulated human neutrophils: conversion of 7S receptor form to the 4S form by GTP γ S. Pooled plasma membrane fraction from unstimulated cells were radiolabeled with FMLPL-SASD- $^{[125I]}$ and solubilized as described in Materials and Methods in the presence (B) or absence (A) of 10 μ M GTP γ S. The extracts were then analyzed by rate zonal ultracentrifugation, followed by SDS-PAGE on 7–20% polyacrylamide gradient gels. The gels were then dried and analyzed by autoradiography. In the autoradiograph (A and B), lanes 1–12 correspond to fractions 1–12 of the gradients; lane 13 is the pellet fraction. (C and D) Sensitivity of G-protein and N-formyl peptide receptor sedimentation rates to exposure to GTP γ S. Western blot analysis simultaneously using two antibodies specific against $G_{i\alpha}$ ($\leftarrow\leftarrow$) and $G_{i\beta}$ (\leftarrow) of the same fractions gradient shown in A and B. $G_{i\alpha}$ and $G_{i\beta}$ distributions are shown for extracts sedimented after exposure to 0 (C) and 10 (D) μ M GTP γ S.

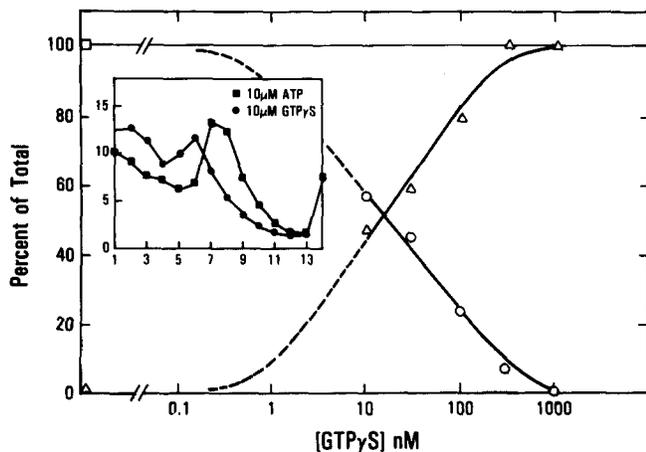


Figure 2. Conversion of N-formyl peptide receptor from the 6-4S form by GTP γ S: nucleotide sensitivity. Plasma membrane extracts were prepared as described in Fig. 1 with the inclusion of 0-10 μ M GTP γ S during solubilization. The total amount of the 7S receptor form (\circ) and 4S form (Δ) are plotted as a function of the log of GTP γ S. (*Inset*) ATP at 10 μ M concentration has no effect on the sedimentation rate of the labeled receptor. The sedimentation profile of 125 I radiolabel is plotted as a function of gradient fraction number for extracts containing (\bullet) 10 μ M GTP γ S and 10 μ M ATP (\blacksquare).

proteins. Occupied receptor-G-protein complexes should be sensitive to GTP and nonhydrolysable derivatives such as GTP γ S. Thus, the solubilization of the membranes was also performed in the presence of 10 μ M GTP γ S and sedimented as described above. Fig. 1 *B* shows that the sedimentation of the receptor was slowed, reducing its apparent sedimentation coefficient to \sim 4S.

To quantitate the GTP sensitivity of the complex, a GTP γ S dose dependency was determined. GTP γ S at six different concentrations ranging from 10 nM to 10 μ M was included in the solubilization and gradient buffers. Fig. 2 shows that the decline of receptor-associated radioactivity in the 6-7S region of the gradient corresponded to an increase in receptor associated radioactivity in the 4S region of the gradient. The 50% effective dose for GTP γ S was \sim 10-30 nM. By contrast ATP had no effect up to a dose of 10 μ M as is shown (Fig. 2, *inset*). These results suggest that the 6-7S form of the receptor may result from a specific association with guanyl nucleotide binding proteins.

Receptor Is Complexed with G-proteins

The presence of guanyl nucleotide binding proteins in the 6-7S receptor fractions was confirmed by Western blot analysis of the same fractions of gradients described in Fig. 1, *A* and *B*. The G-protein was identified using a mixture of two antibodies directed against bovine brain G-protein beta subunit and against the carboxyl terminal nona peptide (NNL-KDCGLF) from the sequence of the alpha subunit of G α (5). Individual application of these antibodies to neutrophil extracts showed no cross-reactivity with the other subunit or other proteins (not shown, see reference 15). Fig. 1 *C* shows that in the untreated sample, the alpha and beta subunits of the guanyl nucleotide binding protein are most enriched in

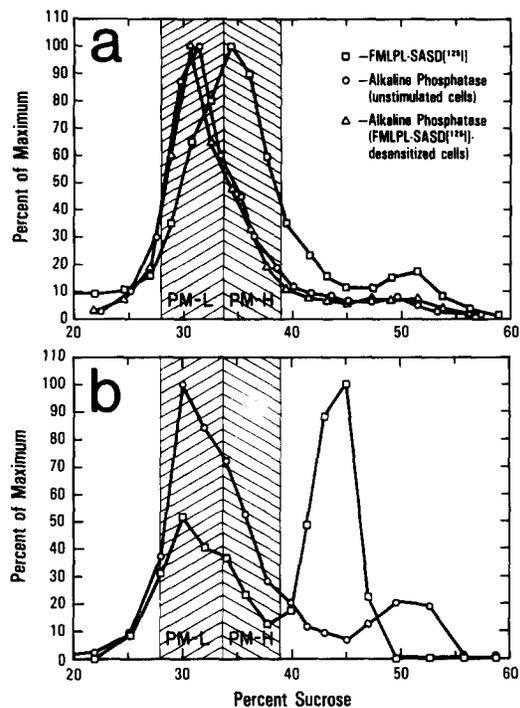


Figure 3. Subcellular distribution of alkaline phosphatase and occupied N-formyl peptide receptor in sucrose density gradient-separated homogenates of control and formyl peptide density-desensitized human neutrophils. Lateral segregation of occupied N-formyl peptide receptors and plasma membrane alkaline phosphatase activity in desensitized human neutrophils is shown in *a*. When cells are desensitized by earlier incubation with FMLP-L-SASD [125 I] and then photoaffinity labeled before N $_2$ cavitation, the distribution of the surface-iodinated receptors (\square) is resolved from the conventional plasma membrane marker alkaline phosphatase (Δ). The distribution of alkaline phosphatase in control cells (\circ , *a* and *b*) is not significantly different from that of desensitized cells. The distribution of N-formyl peptide receptors in fractions obtained from control cells is shown in a separate experiment in *B*. This distribution is obtained when fractions are labeled after N $_2$ cavitation and closely parallels the alkaline phosphatase activity in the regions containing plasma membrane activity. The major peak of receptor is found in the specific granule-Golgi region and results from the unmasking of an intracellular receptor pool.

fraction 6 and 7 but not coincident with the receptor distribution which has a peak in fraction 7.

If receptor and G-proteins indeed form the GTP-sensitive complex that is responsible for the change in the sedimentation properties of photoaffinity-labeled receptor, then there should also be a change in the sedimentation properties of some of the G-protein after GTP γ S treatment. Fig. 1 *D* confirms this prediction, showing that both G α and G β demonstrate a reduction in sedimentation rate after treatment of the extract with GTP γ S. It is noteworthy that their peaks shift from a position of noncoincidence with the receptor peaks to a position of coincidence. This result is not contradictory, if consideration is given to the fact that there are many more G-protein copies in the neutrophil than copies of formyl peptide receptor (5) and furthermore that they are probably coupled or associated to a variety of other receptors in the membrane. After they become uncoupled by application of GTP γ S to the system the cosedimentation of the G-protein subunits with receptor is fortuitous since unasso-

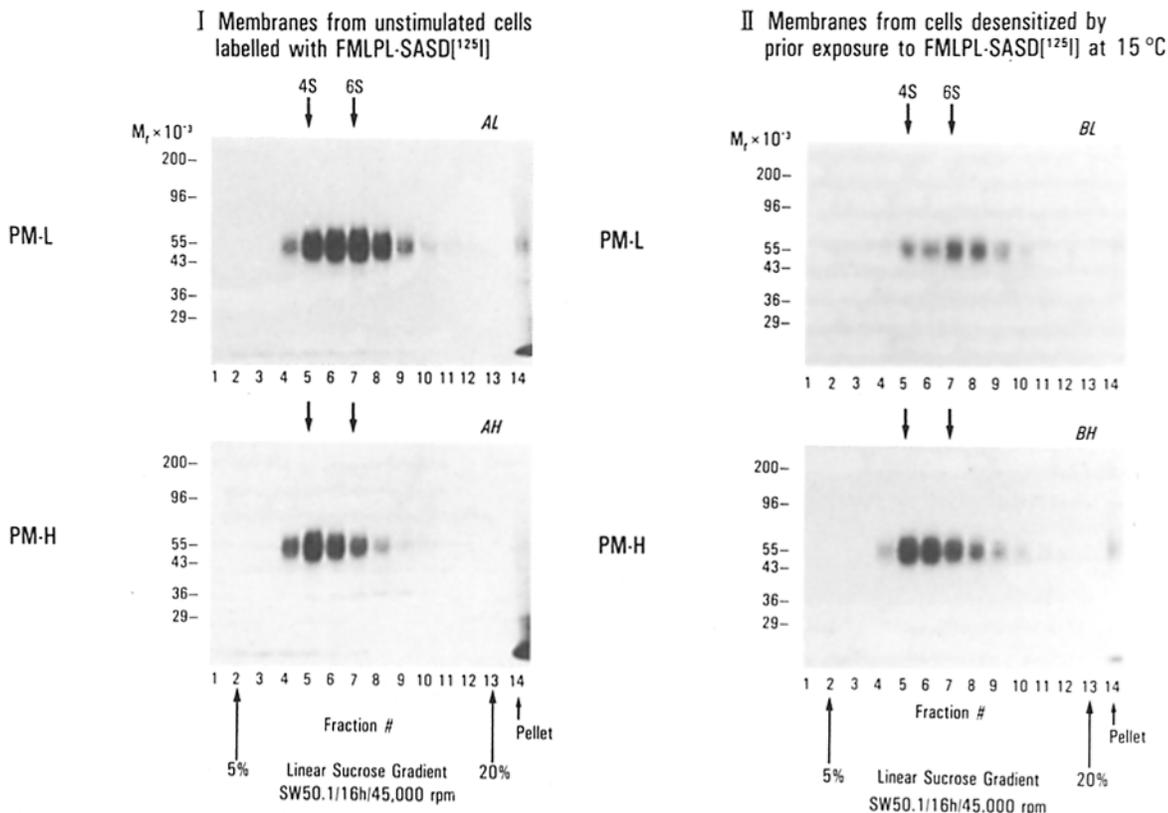


Figure 4. Distribution of the two sedimentible forms of N-formyl peptide receptor in the plasma membrane domains of unstimulated (Column I) and desensitized (Column II) human neutrophils. (Column I) Predominance of the 7S form of the photoaffinity-labeled N-formyl peptide receptor in the light plasma membrane fraction (PM-L) obtained from control cells. Cells received no pretreatment with FMLPL-SASD^[125I]. Membranes were radiolabeled after isolation and separate pooling of light and heavy fractions. Pooled PM-H and pooled PM-L fractions were solubilized in octylglucoside, centrifuged, and fractionated as described in Materials and Methods. Aliquots of each fraction were then analyzed by SDS-PAGE and autoradiography. The autoradiograms are shown for the PM-L (upper) and PM-H (lower) samples. (Column II) Predominance of the 4S form of the occupied N-formyl peptide receptor in the heavy plasma membrane fraction (PM-H) of FMLPL-S-ASD^[125I]-desensitized cells. The cells were pretreated with FMLPL-SASD^[125I] and radiolabeled as intact cells as is described in Fig. 3 A. Fractions were prepared as described above in column I.

ciated alpha subunit of G-protein has a sedimentation coefficient of ~4S (6).

The Receptor Found in Desensitized Cells Is Uncoupled from G-Proteins

Human neutrophils can be desensitized by incubation with N-formyl peptides at lowered temperatures where their normal responses cannot take place; that is 15°C or below. This earlier incubation results in a lateral segregation of N-formyl peptide receptors in the plane of the membrane into cytoskeletal-rich domains depleted in guanyl nucleotide binding proteins. To show that this segregation physically separates all of the receptor population from interaction with G-proteins, we attempted to compare the molecular associations of the occupied and photoaffinity-labeled N-formyl peptide receptors isolated from the light and heavy plasma membrane microdomains, PM-L and PM-H, respectively. We prepared the PM-L and PM-H from cells that had been desensitized by earlier exposure to photoaffinity-iodinated formyl peptide at saturating doses (5 nM) and from cells that had never been exposed to peptide. The isolation of these membranes on continuous isopycnic sucrose density gradients is shown in Fig. 3, *a* and *b*, respectively. Alkaline phosphatase activity,

a marker of the light plasma membrane fraction, has a distribution that is invariant under two conditions, as previously reported (15). In contrast, the occupied formyl peptide receptor is clearly shifted to higher densities in the cells that have been exposed to formyl peptide before their disruption (Fig. 3 *a*).

The distribution of unoccupied N-formyl peptide receptor in the gradients from unstimulated cells is shown in Fig. 3 *b*. In this experiment, the membranes and not the cells (as in Fig. 3 *a*) were photoaffinity labeled after their isolation. The distribution of the 50–70-kD receptor species in between the gradient region containing the granules (40–46%) and endosome/Golgi/cytosol (20–28%) parallels the alkaline phosphatase distribution. The major peak of receptor activity (43–45%) cosediments with specific granule markers. Since it is observed only when photoaffinity labeling is done in broken cell fractions, it represents the intracellular pool of receptors (1, 16).

The light and the heavy fractions from the gradients obtained from both types of cells were then separately pooled as indicated in the hatched areas in Fig. 3. In the case of the membranes prepared from untreated, unlabeled, responsive cells (Fig. 3 *b*), receptors were photoaffinity labeled after

isolation of the membrane as described above. All four pools of membranes (PM-L, PM-H from desensitized cells [Fig. 3 a], PM-L, PM-H from responsive cells [Fig. 3 b]) were then solubilized, fractionated on four detergent-containing velocity sucrose gradients, and analyzed by SDS-PAGE autoradiography as described above. The results are shown in Fig. 4. The receptors extracted from the light fractions sediment most rapidly with an apparent sedimentation coefficient of $\sim 6-7S$ as observed above. The receptors obtained from the heavy fraction sediment slowly with sedimentation coefficient of $\sim 4S$. Thus, in addition to a shift of receptors from the light membrane fraction to the heavy membrane fraction, there appears to be a reduction in receptor sedimentation rate. Since the $4S$ form found in the heavy fraction also does not demonstrate a guanyl nucleotide sensitivity in its sedimentation velocity, then it too is probably unassociated and uncoupled from G-proteins.

Segregated Receptors Can Still Form GTP-sensitive Associations

If occupied receptors found in the heavy plasma membrane fraction were capable of reassociating with G-proteins, then they still might be capable of transducing signals. To assess their potential for interacting with their transduction partners, labeled membranes from the heavy plasma membrane fraction of desensitized cells were solubilized as described above and then mixed with the light fractions of cells treated identically except substituting nonradioactive FMLPL-SASD for the radioactive form. The mixing was done in the presence and absence of $10 \mu M$ GTP γ S. As a control, the heavy fraction was solubilized alone under the same conditions. The extracts were then analyzed as above after velocity sedimentation. Fig. 5 shows that there were approximately twice as many GTP-sensitive, rapidly sedimenting receptors in the mixed sample than in the unmixed control. Application of GTP γ S resulted in a quantitative shift of the reconstituted rapidly sedimenting population (hatched area) to the more homogeneous, slowly sedimenting population (shaded area). This result has been twice reproduced and has also been repeated using membranes that have been washed with 1 M NaCl to remove peripherally bound membrane proteins (not shown). Most recently, the result was confirmed in a separate study using purified bovine G_i to reconstitute a $7S$ receptor form that is fully convertible to a $4S$ form upon application of $10 \mu M$ GTP γ S (Jesaitis, A., J. O. Tolley, and G. M. Bokoch, manuscript in preparation). These results suggest that functional receptors can still be isolated from PM-H of desensitized cells and that these receptors are capable of reestablishing GTP-sensitive associations with available G-proteins.

Discussion

The purpose of this study was to investigate the molecular basis of desensitization and response regulation of chemoattractant-stimulated neutrophils. As a model, comparison was made between the molecular associations of receptor obtained from two previously characterized plasma membrane domains (15) of responsive and desensitized neutrophils. In such desensitized cells, most occupied receptors become segregated from the bulk plasma membrane pool of G-proteins. To invoke this physical separation as a regulatory mech-

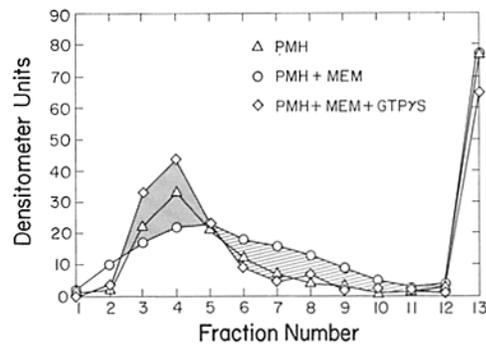


Figure 5. Reassociation of solubilized N-formyl peptide receptors derived from the heavy plasma membrane fraction of desensitized cells with membrane components from the light plasma membrane fraction. Two aliquots of neutrophils (1.5×10^9 cells) were incubated and photoaffinity labeled with either 5 nM FMLPL-SASD or FMLPL-SASD[^{125}I], as described in the legend of Fig. 3. The heavy and light plasma membrane fractions were isolated from each sample. The radiolabeled heavy fraction was then solubilized at 0.3 mg/ml protein concentration in the presence and absence of $10 \mu M$ GTP γ S. After 2 h, nonradioactive membranes from the light fraction were added to the solubilization mixture in the form of a pellet to give a final protein concentration of 2 mg/ml. The mixture was agitated at $4^\circ C$ to solubilize the added membranes for an additional 2 h. Each mixture was then layered on octylglucoside-containing sucrose gradients, centrifuged, and fractionated as described above. The fractions were then run on SDS-PAGE, developed for autoradiography, and quantitated by densitometry. The sedimentation of the radiolabeled receptor is plotted as a function of fraction number from low to high percentage sucrose for receptor without added membrane (Δ) or with added membrane in the presence (\diamond) and absence (\circ) of $10 \mu M$ GTP γ S.

anism controlling the interaction of receptors and G-proteins, it was necessary to show that the receptors that were translocated to the G-protein-depleted domain of the membrane were still capable of associating with G-proteins but were prevented from doing so by their lateral segregation. Our studies now suggest that receptors in desensitized cells are at least in part uncoupled from G-proteins by the fact of their physical separation in the plane of the membrane.

This conclusion is derived from experiments which analyzed the molecular associations of the solubilized N-formyl peptide receptor isolated from different regions of the cell membrane. Such receptors were then compared in hydrodynamic properties when obtained from cells in the responsive and desensitized states. The majority of receptors derived from the light plasma membrane fraction appeared to be associated with guanyl nucleotide binding proteins after solubilization in octylglucoside. In contrast, this association was absent for receptors derived from the heavy plasma membrane fraction. Thus, the G-protein-associated complex was the predominant form only in fully responsive cells but not desensitized cells. We, therefore, conclude that most receptors in unstimulated cells may be preassociated with G-proteins or, alternatively, become associated after labeling (9) or isolation of purified plasma membranes. In contrast, receptors isolated from desensitized cells, are segregated from the bulk plasma membrane pool of G-proteins in a plasma membrane microdomain enriched in actin and fodrin and are isolated in a form that is unassociated with G-proteins. Such receptors, however, still show the propensity for reassociating with a membrane component from the PM-L, potentially

a G-protein, to form a GTP-sensitive molecular complex. We, therefore, infer that the observed cellular desensitization to formylated peptides at reduced temperatures may result at least in part from physical separation of active, functional receptors from their guanyl nucleotide-binding transduction partners, the G-proteins.

Our results also provide some novel information about the subunit structure of the G-protein interacting with neutrophil receptors. Fig. 1 suggests that N-formyl peptide receptor is complexed with G-protein until GTP induces dissociation of the complex. The Western blot analysis identifying the α and β subunits of the G-protein also suggest that both subunits are part of such a complex since they both shift to lower sedimentation rates when the receptor complex is broken up with GTP γ S. It also appears that GTP affects the integrity of only a fraction of the total G-protein pool (presumably only those associated with occupied receptors). This result supports the concept that there are excess G-proteins in the membrane presumably unassociated or interacting with other proteins (7).

We believe that the molecular events leading to the "non-physiological" desensitization at 15° is of central importance to the normal functioning of the human neutrophil and its activation by formyl peptides. As stated in the introduction, our evidence and that from other laboratories suggests that the reduced temperatures in these experiments serve to block internalization or removal of the occupied receptors from the surface of the neutrophil. This block permits the molecular dissection of this normally transient state of receptor processing. Indeed, after Pertussis toxin treatment of cells, receptors are uncoupled from ribosylated G-proteins and are found in their high affinity guanyl nucleotide-insensitive state (23). Receptor coisolation with the cytoskeleton is increased in such cells as is the fraction of 4S receptors measured by the sedimentation analysis of detergent extracts of membranes from Pertussis toxin-treated cells (Jesaitis, A., and J. Tolley, unpublished results). Thus, our current results support this hypothesis and suggest that response termination is at least partially controlled by high affinity complex formation and lateral segregation of receptors from their G-protein transduction partners.

Studies using other sensory systems have indicated that receptor phosphorylation also serves to decrease receptor efficacy or coupling to G-proteins and thus may also be an alternative or simultaneous regulatory step in formyl peptide receptor-mediated signal transduction (21, 22). Phosphorylation of dictyostelium discoideum cAMP chemoattractant receptors appears to account for adaptation to persistent stimulus presentation and thus decreased transduction (18). Growth factor receptors, such as the epidermal growth factor receptor, also become phosphorylated (see reference 21 for review). The phosphorylation at threonine and serine sites by protein kinase C appears to reduce occupied receptor kinase activity, decrease receptor affinity for its ligand, and promote receptor internalization. However, this type of receptor inactivation does not appear to completely account for response termination or control. Phosphorylated rhodopsin receptors (28) also demonstrate reduced functional coupling to guanyl nucleotide-binding proteins but require interaction with a 48-kD protein called arrestin to achieve full deactivation (27). In the β -adrenergic system phosphorylated receptors

from membranes of desensitized cells were not completely inactivated as judged by their partial ability to reconstitute activation of adenyl cyclase activity (17).

Thus, receptor sequestration by binding to inhibitor molecules such as arrestin, by internalization, or lateral segregation, appears to play a crucial role in response regulation in most of these systems. Indeed, there may be several levels of control that are required to ensure full fidelity of the regulation, i.e., complete turn off of occupied receptors. Such control may be essential to the function of the cell and its ability to alter the level of its sensitivity through the adaptive process.

For the human neutrophil system, the implications of organizational control of receptor G-protein interaction are of particular interest. One such implication is that any perturbation of membrane order could have a major effect on the interaction of receptors with G-proteins. Thus, the potentiating effects of plasma membrane or cytoskeletal perturbants such as lectins, lipids, or cytochalasins could be explained by increased interaction of receptor and G-proteins due to interference with the mechanism of termination of the response by lateral segregation of receptors and transducers. In addition, pathological states in the host which interfere with such regulation might promote inappropriate activation or loss of response regulation which would then contribute further to the pathology.

We gratefully thank Velda Comstock and Elizabeth Escher for their excellent typing assistance and Dr. M. T. Quinn for helpful suggestions.

We acknowledge support of this work with United States Public Health Service grants AI17354, RO1 AI22735, and PR00833. A. J. Jesaitis and G. M. Bokoch are recipients of American Heart Association Established Investigator Awards and Grants-in-Aid with funds contributed in part by the California affiliate of the American Heart Association.

Received for publication 30 May 1989 and in revised form 21 August 1989.

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