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The Fate of the N-Formyl-Chemotactic Peptide Receptor in Stimulated Human Granulocytes: Subcellular Fractionation Studies

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Experiments were performed to examine how human granulocytes, stimulated by N-formyl-chemotactic peptides, process the N-formyl peptide receptor. One percent of the surface N-formyl-chemotactic peptide receptors of purified human granulocytes were covalently, specifically, and radioactively labeled at 4°C using the photochemically reactive N-formyl-chemotactic hexapeptide CHO-Nle-Leu-Phe-Nle-[^{125}I] Tyr-N$^{\epsilon}$(6-(4′-azido-2′-nitrophenyl-amino)hexanoyl)-Lys. After incubation in the presence of 500 nM of N-formyl-Met-Leu-Phe at 37°C, the cells were lysed and fractionated by isopycnic surcrose density gradient sedimentation. Receptor-associated radioactivity cosedimented with plasma membrane in fractions from cells kept at 4°C or incubated at 37°C for 2 min or less. Fractionation of cells incubated at 37°C for longer times revealed that the radioactivity sedimented to lower densities coincident with Golgi markers and the site of noncovalently bound and internalized formyl-chemotactic peptide. To follow the redistribution of unoccupied receptors, human granulocytes were stimulated with 500 nM N-formyl-Met-Leu-Phe at 37°C for 5 min, washed, lysed by N_2 cavitation, and fractionated by rate zonal sucrose density gradient sedimentation. Compared to unstimulated controls the specific binding of N-formyl-Met-Leu-[^{3}H]Phe decreased 76% ± 9% in plasma membrane fractions. N-formyl-Met-Leu-[^{3}H]Phe-binding activity associated with an intracellular pool cosedimenting with specific granules remained unchanged. Approximately 20% of the activity lost in the plasma membrane could be accounted for by a redistribution of specific N-formyl-Met-Leu-Phe binding to fractions enriched in azurophil granules. We conclude that the receptor is the carrier in the internalization of the N-formyl-chemotactic peptides to a Golgi-enriched fraction and hypothesize that after a short residency

Abbreviations used: CP, N-formyl methionylleucylphenylalanine; EDTA, ethylene diaminetetraacetica-cid; WGA, wheat germ agglutinin; ^{125}I-WGA, ^{125}Iodine-conjugated wheat germ agglutinin; NPH ^{125}I-peptide; CHO-Nle-Leu-Phe-Nle[^{125}I]Tyr-N$^{\epsilon}$(6-(4′-azido-2′-nitrophenyl-amino)hexanoyl)-Lys.

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in this fraction, the receptor may dissociate from the ligand and pass onto a fraction cosedimenting with dense granules.

Key words: N-formyl-chemotactic peptide, granulocytes, subcellular fractionation, peptide receptors, endocytosis

Leukocytes can maintain prolonged directed migration (chemotaxis) along gradients of chemotactic factors using mechanisms which are triggered by the binding of the factor to specific cell-surface receptors [1]. The binding is followed by sensory tranduction events which eventually result in directed motility. Among the complex biochemical interactions which appear to regulate the sensitivity of the cell to chemotactic factors is the insertion or removal of receptors at the cell surface [2,3].

Granulocytes have the ability to accumulate chemotactic factors internally to levels which significantly exceed the original numbers of receptors expressed at the cell surface, even in the presence of inhibitors of protein synthesis [4–5]. To explain the continued sensitivity of the cells in chemotactic gradients under these conditions, receptor reuse (recycling) or expression of new receptor from cellular pools has been postulated [6–8] and measured indirectly [3,4].

Compartmentalization of the internalized formyl-chemotactic peptide has now been analyzed in our laboratory, and the evidence suggests that the peptide passes through a Golgi-enriched subcellular fraction and is accumulated in the cytosol [9]. There is, however, no direct evidence that the receptor is the carrier in this process. Moreover, the fate of the receptor after peptide internalization is unknown.

In this paper, we report our studies of the subcellular distribution of the N-formyl-chemotactic peptide receptor before and after stimulation of human granulocytes with saturating doses of N-formyl-Met-Leu-Phe. We have followed the internalization of the receptor covalently labeled with a photoaffinity derivative of an N-formyl-chemotactic hexapeptide and have measured the subcellular distribution of unoccupied receptors in cell lysates fractionated by sucrose density gradient sedimentation. We conclude that the occupied receptor is internalized into a Golgi-enriched subcellular fraction and speculate as to its subsequent fate in the cell.

MATERIALS AND METHODS

The method of granulocyte isolation, cavitation, fractionation, marker analysis, and the materials and buffers used are described elsewhere [8,9] and are summarized in Figure 1. Briefly, granulocytes were isolated from whole human blood to 98–99% purity and surface labeled with trace amounts of ^{125}I-WGA wheat germ agglutinin (^{125}I-WGA), washed, and maintained at 4°C in cell resuspension buffer [10] containing 0.1% bovine serum albumin.

Experiments were performed in which cells were stimulated at 37°C with N-formyl-Met-Leu-Phe (CP) in cell buffer that was bubbled with argon or nitrogen and supplemented with 0.1% bovine serum albumin, 10 μg/ml of superoxide dismutase, and 7μg/ml catalase (Sigma Chem Co, St Louis) to minimize deleterious effects of superoxide anion production by the cells. The suspensions were washed, resuspended in 0.34 M sucrose, 1mM EDTA, 1 mM Na_2ATP, 1 mM dithiothrietol 0.1 mM $MgCl_2$ and 10 mM Hepes buffer, pH 7.4, at a cell concentration of 2–8 $\times$ 10^7 cells/ml, lysed by N_2 cavitation (400 psi/15 min/0°C), and fractionated by sedimentation at 1,000g for 5 min into a low-speed supernatant and pellet-foam residue. The low-speed

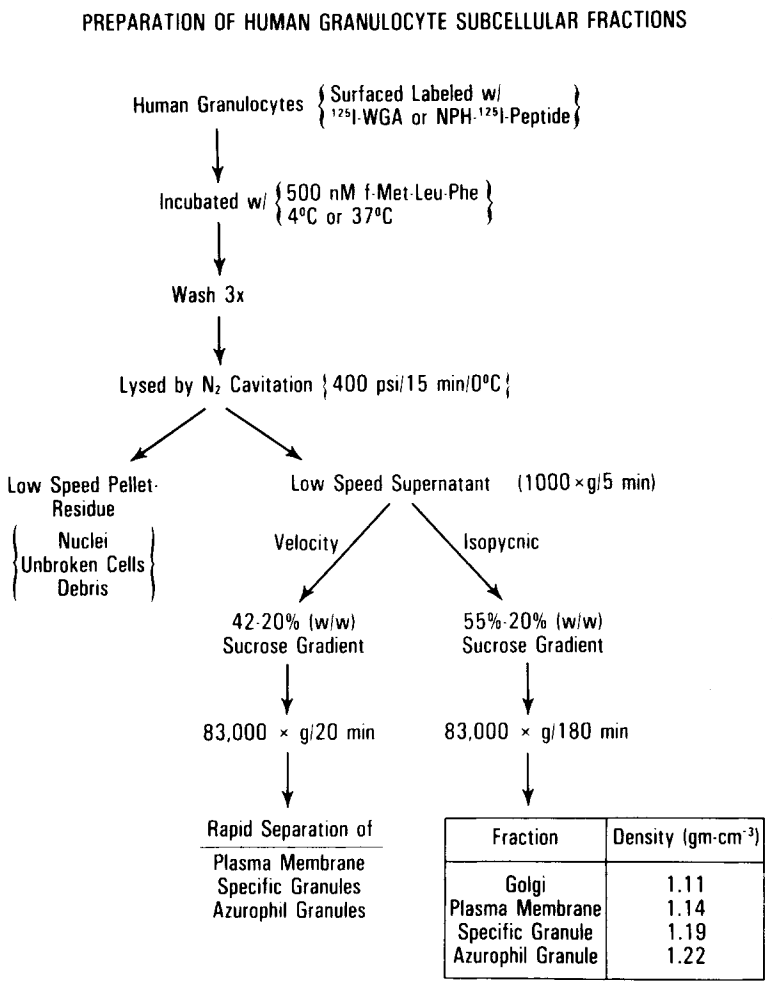

Fig. 1. Summary of subcellular fractionation protocol.

supernatant was used for further analysis and separated by either velocity (42–20% [w/w] sucrose) or isopycnic (55–20%) sucrose density gradient sedimentation at 4°C in a Beckman SW27 (25,000 rpm; 20 min or 180 min) or SW41 rotor (40,000 rpm, 120 min). One point-five-milliliter or 0.75-ml fractions were collected from the respective gradients. Further details of each experiment are given in the figure legends or text. Average values are given with standard deviations and number of trials in parenthesis.

Specific CP-binding activity was measured using N-formyl-Met-Leu-[^{3}H]Phe (^{3}H-CP) from New England Nuclear Corp and 0.22-μm Millipore cellulose acetate filters by the method of Tsung et al [11]. The free concentrations of ligand used to measure total and nonspecific binding were 14.1 nM of ^{3}H-CP $\pm$ 1 μM of nonradioactive CP. Nonspecific binding was 250 $\pm$ 150 cpm. ^{125}I-WGA was prepared following Cuatrecasas [12] to a specific activity of 3×10^7 cpm/μg and used to surface label cells to a maximum activity of 10^6 cpm/10^8 cells. These levels were much below

agglutinating concentrations and did not interfere with normal stimulation of superoxide anion production, CP binding, or any of the activities described herein. Marker-enzyme activities used for subcellular analysis were β-glucuronidase [13], lysozyme [14], UDP-galactose galactosyl transferase [15] with ovomucoid as the acceptor. Protein was determined by the method of Bradford [16] using the "Bio-Rad" Protein Assay kit with bovine serum albumin as a standard.

In situ photoaffinity labeling of the human granulocyte N-formyl-chemotactic peptide receptor was carried out as modified from Painter et al [17]. 2.5×10^8 purified human granulocytes were prepared and resuspended (2.5 ml) in protein free cell buffer [10]. The cells were divided into two equal aliquots and one aliquot preexposed to 100 nM N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys for 10 min at 4°C to block binding of the photoaffinity derivative at subsequent steps. To both, 5 nM (8×10^6 cpm total) CHO-Nle-Leu-Phe-Nle-[^{125}I] Tyr-N$^{\epsilon}$(6-(4′-azido-2′-nitrophenyl-amino) hexanoyl)-Lys (NPH ^{125}I-peptide) was added. The cells were maintained in the dark for 15 min on ice in sealed borosilicate glass tubes purged with N_2. The tubes were irradiated at 360 nm in a Rayonet UV light illuminator for 2 min and the cells washed two times in cell buffer containing 0.1% bovine serum albumin. The cells were then resuspended to a cell concentration of 2×10^7 cells/ml in cell buffer containing albumin, catalase, superoxide dismutase, 500 nM CP, and divided into two equal volumes again. Two aliquots (blocked control and unblocked) were brought to 37°C and incubated for 10 minutes. The remaining two aliquots were maintained at 4°C. The cells were then washed and fractionated as described above.

Because the majority of the radioactivity associated with the cell was noncovalently attached, the protein-associated and hence receptor-associated [17] radioactivity was determined following a modification of Nordeen et al [18]. One hundred to 300 μl of fraction was spotted onto a Whatman G/F glass fiber filter and dried. The filters were then immersed and washed in ice-cold trichloroacetic acid (TCA) and washed again in ice cold acetone. The residual radioactivity was counted in a Baird-Atomic gamma counter.

RESULTS

Fractionation Characteristics

Isolated granulocytes were surface labeled with ^{125}I-WGA and stimulated at 37°C with N-formyl-Met-Leu-Phe in order to prepare a population of stimulated cells for subcellular fractionation and comparison with fractions from unstimulated cells. Such populations were lysed by N_2 cavitation and fractionated on sucrose density gradients. Recoveries of protein, ^{125}I-WGA (plasma membrane), galactosyl transferase (Golgi), and lysozyme (granules) after N_2 cavitation were the same for both prestimulated and unstimulated cells, as well as for cells always kept at 0–4°C. The complete analysis of subcellular fractionation and legitimacy of ^{125}I-WGA as surface marker is presented in Jesaitis et al, [8,9]. Approximately 80% of cellular protein and the ^{125}I-WGA surface label was recovered, of which 40–50% was in the low-speed supernatants. No DNA was detectable in the low-speed supernatants. The distribution of markers for granules, endoplasmic reticulum, mitochondria, Golgi, were similar for lysates obtained from both stimulated and unstimulated cells (kept at 0–4°C or 37°C) in velocity and isopycnic [9] sedimentation runs. The ^{125}I-WGA profiles for these gradients were also unaffected by keeping unstimulated prelabeled

cells at 37°C for 5 min. Minimum recovery of the marker activities on sucrose gradients was 75% of the amounts in the low-speed supernatants [9].

Internalization of the N-Formyl Chemotactic Receptor by Granulocytes—Subcellular Fractionation Studies

Recently, we have shown that if granulocytes are incubated with 50 nM ^{3}H-CP at 37°C for various times, then after 1 min radiolabel is transferred from the plasma membrane to a subcellular compartment distinct from plasma membrane and cosedimenting with the low-density component of the Golgi marker, galactosyl transferase [9]. No significant amounts of radioactivity were found in the granule or lysosomal fractions.

Is the receptor the carrier in this process? An experiment was performed in which the peptide receptor was cross-linked to a photochemically reactive and radiolabeled aryl azido derivative of an N-formyl-chemotactic hexapeptide (NPH ^{125}I-peptide) in resting cells kept at 0–4°C. Control cells were prepared by blocking the photoinduced cross-linking with the addition of 20-fold excess of underivatized nonradioactive peptide. The labeled and blocked cells were incubated at both 4°C and 37°C for 10 min and then washed, lysed by N_2 cavitation, and fractionated by isopycnic sucrose density gradient sedimentation. Each fraction was analyzed for TCA-precipitable radioactivity, which uniquely represented affinity-labeled receptor [17]. After fractionation, 90–95% of the radioactivity incorporated into the cells was recovered; 60–70% of the TCA-precipitable radioactivity in the 1,000*g* supernatant was recovered in the gradients.

The upper panel of Figure 2 shows the distribution of TCA-precipitable radioactivity in the sucrose gradient fractions from labeled cells (unblocked) plotted as a function of percent sucrose. The two curves show the distribution profiles for the cells kept at 4°C and 37°C. The profile from the 4°C cells shows that the photolabeled receptors in the subcellular fractions band at a sucrose density (34% ± 1% [w/w]; n = 5 fractionations) corresponding to the plasma membrane-enriched region [9] of such gradients (peak at 32.3% ± 1.3%; n = 14). The middle panel shows the distribution of radioactivity in fractions obtained from lysates of blocked control cells and shows that the radioactivity in the cytosol fractions (<20% sucrose) is nonspecific. When lysates of labeled cells, *incubated at 37°C* for 10 min, were fractionated, a major shift in the sedimentation profile of the radioactivity to lower densities (27% ± 1% sucrose; n = 5) was observed. We have now shown [8,9] that these densities correspond to the position of the low-density component of the Golgi marker galactosyl transferase (25% ± 1% sucrose; n = 5). In addition, they also correspond to the position of the internalized and membrane associated ^{3}H-CP which is not covalently bound [9]. For comparison, two distribution profiles obtained from fractionations of cells incubated with ^{3}H-CP at 37°C for 1 min (no internalization) or 10 min (significant internalization) are shown in the lower panel. The evidence in Figure 2 suggests, therefore, that the N-formyl-chemotactic receptor which has been covalently coupled to its ligand is internalized within 10 min into the same subcellular fraction as is the chemotactic peptide N-formyl-Met-Leu-Phe. The receptor and the peptide cosediment with the Golgi marker galactosyl transferase. For reference, the position of the granules, Golgi, and plasma membrane in the gradient are marked by arrowheads in the upper panel.

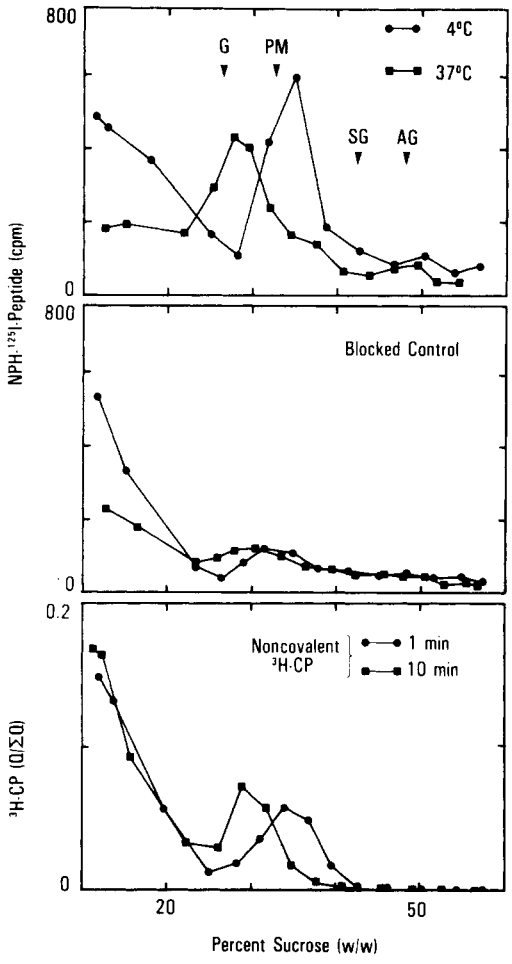

Fig. 2. Isopycnic sucrose gradient analysis of lysates from NPH ^{125}I-peptide-labeled granulocytes. Upper) Sucrose gradient profiles of fractionated lysates from 2.5 × 10^8 granulocytes that were prelabeled with NPH ^{125}I-peptide in the absence of underivatized peptide. Each batch (1.25 × 10^8) of cells was exposed to 500 nM N-formyl-Met-Leu-Phe and incubated at 37°C (■—■) or at 4°C (●—●) for 10 min before N_2 cavitation and fractionation. NPH-^{125}I-peptide content (cpm/0.1 ml) of each gradient fraction (1.5 ml) is plotted as a function of percent sucrose (w/w). The fractions having the maximum content of low-density Golgi (G), plasma membrane (PM), specific granules (SG), and azurophil granules (AG) are indicated by arrowheads. Middle) Profiles of fractionated lysates from granulocytes labeled with NPH-^{125}I-peptide in the presence of 20-fold excess underivatized peptide (blocked control). Experiment performed and displayed identically as in upper panel. Lower) Profiles of fractionated lysates from granulocytes exposed to 50 nM N-formyl-Met-Leu-[^{3}H]Phe (noncovalent) at 37°C for 10 min (■—■) and for 1 min (●—●). The fractional content of N-formyl-Met-Leu-[^{3}H]Phe (^{3}H-CP) recovered in each gradient fraction (Q/ΣQ) is plotted as a function of percent sucrose (w/w). Recalculated from Jesaitis et al [9].

Our previous studies have shown that the internalized N-formyl-Met-Leu-Phe resides in the Golgi-enriched fraction transiently and eventually passes into the cytosol fraction. In order to follow the processing of the internalized *receptor* further, the subcellular fractionation was performed after 2, 20, and 40 min of incubation of the NPH ^{125}I-peptide-prelabeled cells with 0.5 μM N-formyl-Met-Leu-Phe at 37°C. These profiles are shown in Figure 3. When cells were incubated for only 1 min at 37°C or at 4°C, the organelle-associated radioactivity in the sucrose gradients was found in the plasma membrane-enriched region (also see Fig. 2). At later times, the majority of the sedimentable (ie, organelle-associated) radioactivity was found in the low density region of the gradient corresponding to the Golgi-enriched fractions. No significant decrease in the radioactivity in these fractions was observed at the later times, indicating that the labeled receptor remained in this compartment. Only a small increase of radioactivity was observed at 20 and 40 min in the dense granule fractions.

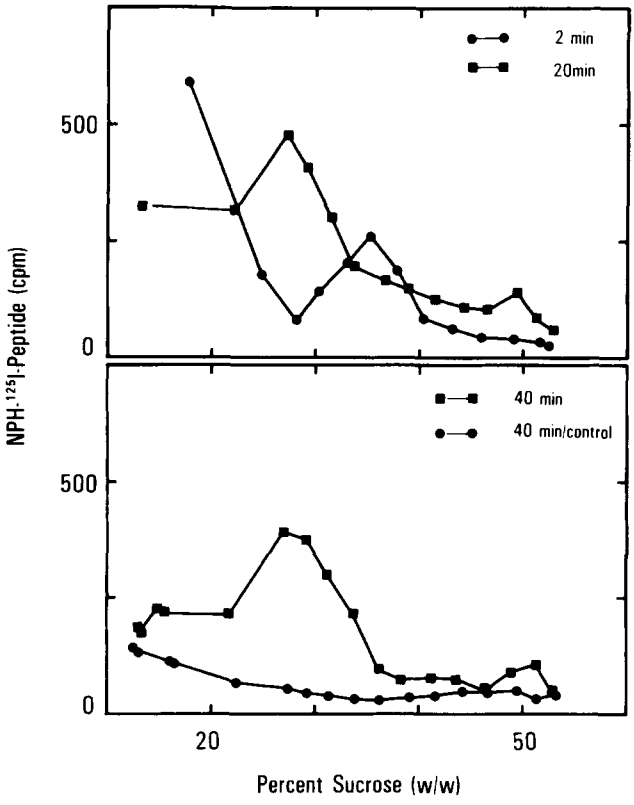

Fig. 3. Kinetics of receptor redistribution in subcellular fractions after stimulation of granulocytes by 500 nM N-formyl-Met-Leu-Phe. Cells (5×10^8) were prelabeled with NPH-^{125}I-peptide and then incubated with 500 nM N-formyl-Met-Leu-Phe for 2 min, 20 min, and 40 min. Blocked control cells were kept at 37° for 40 min. Each batch was washed, lysed by N_2 cavitation, and fractionated by isopycnic sedimentation on linear sucrose gradients. TCA-precipitable radioactivity is plotted (cpm/0.3 ml) as function of percent sucrose.

Redistribution of N-Formyl-Chemotactic Peptide-Binding Activity After Stimulation

One disadvantage of the photoaffinity method is that it does not mark unoccupied, functional receptors. In order to detect these receptors, a CP-binding assay was carried out on subcellular fractions separated by velocity sedimentation. Sucrose density gradient centrifugation at $83,500g_{av}$ for 20 min (velocity) was carried out on the 1,000g supernatant fraction from both stimulated (500 nM CP for 5 min at 37°C) and unstimulated (control: 5 min at 37°C, no CP) cells. Figure 4 shows a representative (one of three experiments) velocity sedimentation analysis of two identical gradient separations of lysates derived from cells of a single donor and then divided into two for stimulation (S) and control (U). The total protein recovered from both gradients was approximately the same (6.4 and 6.8 mg from U and S, respectively).

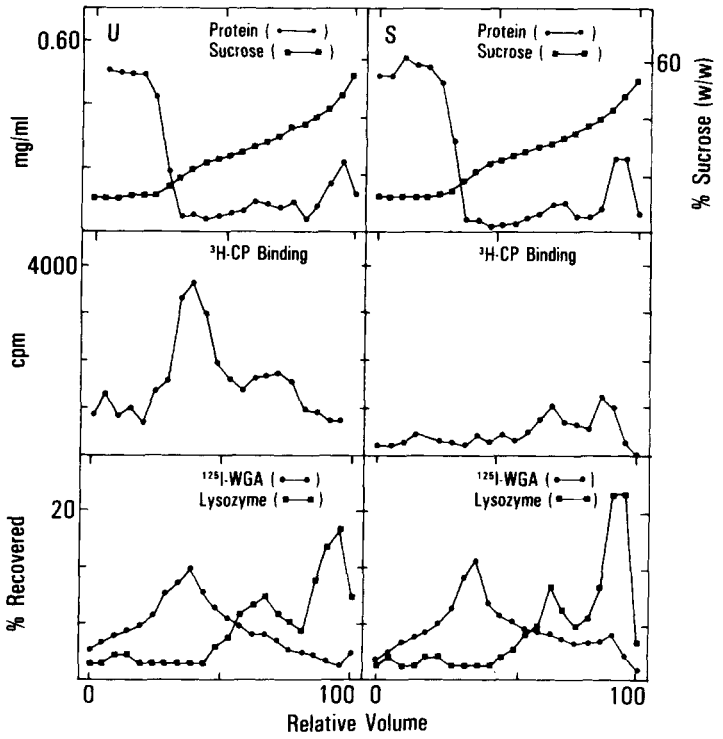

Fig. 4. Redistribution of N-formyl-Met-Leu-Phe (CP)-binding activity in subcellular fractions from human granulocytes stimulated with 500 nM CP. 4.6×10^8 cells were prelabeled with 11×10^6 cpm ^{125}I-WGA and divided into two equal aliquots. One aliquot was exposed to 500 nM CP (S) for 5 min at 37°C. The control was kept at 37°C for 5 min without CP (U). The suspensions were then washed twice in cell-resuspension buffer, once in cavitation buffer, resuspended in 14 ml of the latter, and successively lysed by N_2 cavitation. 12 ml of the low-speed supernatant (1000g, 5 min) was recovered (3×10^6 cpm), diluted to 14 ml, and layered (13 ml) onto a SW 27 sucrose gradient, and fractionated. Marker activities were plotted as a function of relative volume collected. The left panel (U) shows fractions from unstimulated cells; the right panel (S) shows fractions from stimulated cells. Top) Percent sucrose and protein concentration. Middle) ^{3}H-CP (N-f-Met-Leu-[^{3}H]Phe)-binding activity per 100 μl of fraction. Bottom) ^{125}I-WGA and lysozyme content.

The plasma membrane surface marker was distributed in a unimodal but broad profile with the peak just entering into the gradient. Lysozyme, a marker for granules, distributed bimodally into a rapidly sedimenting band in the densest region of the gradient and a slowly sedimenting band at intermediate densities.

The velocity and isopycnic sedimentation profiles of lysozyme accurately reproduce the results of Bretz and Baggiolini [19] who have identified the granule constituents. We have confirmed that the rapidly sedimenting and high density (in isopycnic gradients) lysozyme peak cosediments with β-glucuronidase activity (data not shown) and that the two fractions have the morphologies previously described [19]. These two fractions probably correspond to azurophil (rapidly sedimenting, high density) and specific (slowly sedimenting, intermediate density) granules.

The major difference between stimulated and unstimulated fraction was observed in the formyl-chemotactic peptide (CP)-binding activity. Table I summarizes the comparison of peptide-binding activity in the two gradients and provides the values necessary to calculate the change in binding on a per mg protein basis. The specific activity of CP-receptor sites dropped from 22 pmol/mg protein to 3.2 pmol/mg in the peak ^{125}I-WGA-containing fractions (plasma membrane) from the unstimulated and stimulated cell lysates, respectively. The total activity recovered from these gradients represents 14.7 and 4.7 pmol of receptor-binding sites for these two respective cases. When corrected for the equivalent number of cells fractionated on the gradients (from protein and ^{125}I-WGA recovery), these numbers are equivalent to 9×10^4 and 3×10^4 sites/cell, respectively. These activities were calculated based on an N-formyl-Met-Leu-Phe-receptor dissociation constant of 5.8 nM measured by Scatchard analysis on isolated plasma membranes. In control experiments which measured the total sedimentable binding activity obtained from the cavitation lysates of cells exposed to ± 500 nM CP but at 4°C, no significant differences were observed. This result indicated that the loss at 37°C was not due to incomplete washing.

There was also an overall drop in activity in the more slowly sedimenting granule region (low density lysozyme) and development of a small peak in binding activity of the most rapidly sedimenting granule fractions (high density lysozyme peak) upon stimulation. It is important to note, however, that a significant fraction of the decreased binding activity in the specific granule region probably results from the loss of the underlying plasma membrane binding activity which contaminates the granule fractions as evidenced by their ^{125}I-WGA content. In both gradients there is a shoulder in the "slow" granule fraction whose integrated size is not significantly changed above a plasma membrane baseline activity. Furthermore, although the specific activity of receptor binding in the rapidly sedimenting granule fractions remains relatively constant, the change in content becomes more evident with evaluated as an increase above "baseline."

The changes in plasma membrane marker and chemotactic peptide-binding activity profiles after stimulation with 500 nM CP are summarized in Table II. The ratio of specific activities on an mg protein basis (stimulated/unstimulated) of these quantities in the peak fractions of plasma membrane, slow granules (specific), and fast granules (azurophil) were averaged from four experiments (five donors) and tabulated. The specific activity of the plasma membrane marker remains approximately the same after stimulation in both the plasma membrane peak and the slowly sedimenting lysozyme peak, but doubles in the "fast" granule region. The chemotac-

TABLE I. Down-Regulation of N-Formyl-Met-Leu-Phe Receptors*

Peak fraction	Gradient	B × 10^{-3} (cpm)	B_o(pmol)	M (mg protein)	B_o/M (pmol/mg)
Plasma membrane (^{125}I-WGA)	U	54.6	1.68	0.075	22.4
	S	6.3	0.19	0.060	3.2
Specific granules ("slow" lysozyme)	U	24.7	0.76	0.128	5.9
	S	10.4	0.32	0.090	3.5
Azurophil granules ("fast" lysozyme)	U	10.8	0.33	0.240	1.4
	S	15.5	0.48	0.350	1.4

*Calculation of number of receptor sites B_o and their specific activity B_o/M from the data of Figure 4. From binding equilibria: $B_o = (B/\gamma)(1 + K_D/F)$ where B = amount specifically bound (cpm); K_D = ligand receptor-dissociation constant = 5.8 nM derived from Scatchard analysis for this preparation; F = free ligand concentration used in assay = 14.1 nM; γ = specific radioactivity of N-formyl-Met-Leu-[^{3}H] Phe = 4.6×10^4 cpm/pmol; M = mass of protein (mg).

TABLE II. Relative Change in N-Formyl-Chemotactic Peptide-Binding Activity in Subcellular Fractions From Stimulated Cells as Compared to Unstimulated Cells

Gradient fraction	Specific activity ratio[a]	
	^{125}I-WGA	^{3}H-CP binding
Plasma membrane	0.9 ± 0.3	0.24 ± 0.09
"Slow" granules	1.0 ± 0.3	0.50 ± 0.2
"Fast" granules	2.0 ± 0.4	0.83 ± 0.13

[a]Ratio (fractions from stimulated cells/fractions from unstimulated cells) of specific activities (cpm/mg protein; ^{125}I-WGA or N-formyl-Met-Leu-[^{3}H]Phe) from four experiments using five donors in which activities were measured in parallel. Errors are standard deviations.

tic peptide-binding activity, however, decreases by 76% in the plasma membrane, 50% in the slow granule region and remains approximately the same in the "fast" granule fractions. These results suggest that receptor activity is lost (down-regulated) in the plasma membrane fraction but not to the same degree in the granule fractions. Because there is also lack of correspondence of the distribution profiles of N-formyl-Met-Leu-Phe-binding activity and ^{125}I-WGA content, we suggest that the evidence supports the existence of an independent nonplasma membrane pool of receptor in the slow granule fraction. Finally, the changes in the "fast" granule fractions imply that plasma membrane constituents (including some receptor) is redistributed to the fast granule fraction after stimulation. If this redistribution is interpreted as new activity in this region of the gradient, it would account for approximately 20% (from Fig. 4) of the activity lost from the plasma membrane fraction.

Redistribution of ^{125}I-WGA

^{125}I-WGA is an excellent marker for plasma membrane in granulocytes, as evidenced by its cosedimentation with other surface markers such as 5′-nucleotidase

[9], Na^+K^+ATPase (Jesaitis, unpublished), and surface-labeled N-formyl-chemotactic peptide receptor. ^{125}I-WGA also demonstrated a similar reproducible small redistribution and increase in the dense granule fractions on isopycnic gradients. This rapidly sedimenting and dense ^{125}I-WGA activity increased with the time of incubation of cells with CP, and the accumulation after 25 min at 37°C is shown in Figure 5. The accumulation could result from either a nonspecific aggregation of plasma membranes to dense grandules after cavitation or a specific cellular process in which plasma membrane glycoproteins are internalized into a dense "lysosomal" compartment. Additional evidence presented below suggests that the latter hypothesis is more likely and is discussed in the Discussion section. It is important to note here that this redistribution of ^{125}I-WGA is not readily detected in the absence of CP even when the prelabeled cells are incubated at 37°C for up to 10 min.

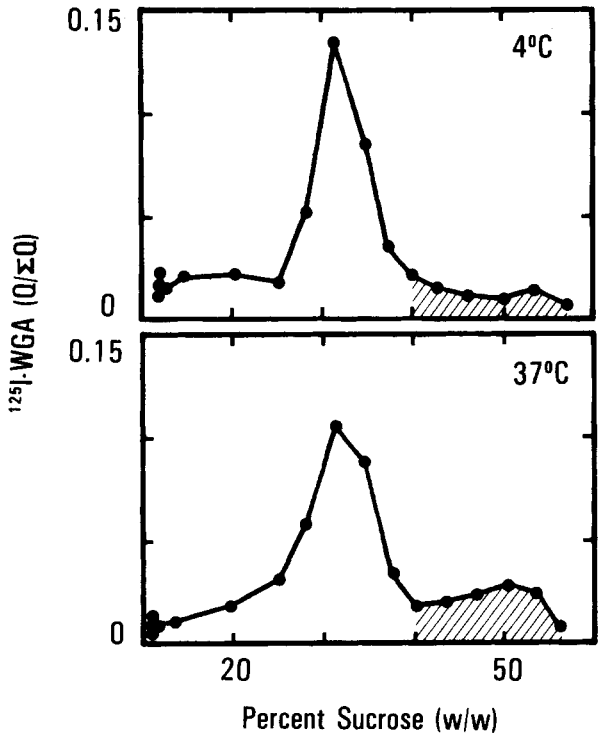

Fig. 5. Plasma membrane marker redistribution in subcellular fractions after stimulation of granulocytes by 50 nM N-formyl-Met-Leu-Phe. Cells prelabeled with trace amounts of ^{125}I-WGA were exposed to 50 nM N-formyl-Met-Leu-Phe at 4°C (upper) and 37°C (lower) for 25 min. Each batch was then washed, lysed by N_2 cavitation, and fractionated. Isopynic sucrose density gradient profiles of the fractional ^{125}I-WGA content of each gradient fraction (Q/ΣQ) are plotted as a function of percent sucrose.

DISCUSSION

The experiments described in this work were designed to examine the redistribution of the N-formyl-chemotactic peptide receptor after exposure of granulocytes to N-formyl-Met-Leu-Phe (CP) in order to gain insight into the way granulocytes process the receptor-peptide signal after binding of the stimulating peptide. In parallel studies [9], we have demonstrated that CP is internalized into a Golgi enriched fraction via a high-affinity, high molecular weight complex first formed in the plasma membrane. Our results here suggest that after exposure of granulocytes to N-formyl-Met-Leu-Phe for 5 min, chemotactic peptide *receptor* is lost from plasma membrane or undergoes changes in its binding parameters, causing a decrease in receptor binding activity in plasma membrane. Most of this loss probably results from internalization of the *receptor-ligand complex* into a Golgi-enriched fraction. Covalently coupled receptor-ligand complex is lost from this latter fraction very slowly (>hr) if at all. In addition, there also appears to be some transfer of unoccupied receptor to the dense granule-enriched fractions. This transfer parallels translocation of plasma membrane glycoproteins and a small percentage of covalently coupled receptor.

Internalization of the Receptor: Covalent Cross-linking Studies

Covalent cross-linking of the N-formyl-chemotactic peptide receptor to a stimulatory photoaffinity derivative of N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys can be achieved in cells at low temperature without significantly altering their viability or responsiveness. The plasma membrane- and protein-associated radioactivity is specifically coupled to the formyl-chemotactic receptor [17]. Under conditions of the experiments reported here, 1% of the total receptors at the cell surface were labeled. These receptors were specifically and quantitatively transferred from the plasma membrane fraction to a Golgi-enriched fraction after approximately 2 min stimulation with underivatized formyl peptide at 37°C. This transfer directly parallels what is observed with radiolabeled formyl peptide that has *not* been covalently coupled to the receptor. We conclude, therefore, that the receptor is probably the carrier in the peptide internalization process.

N-Formyl-Chemotactic Peptide Receptor "Down-Regulation"

We have also shown that receptor-binding activity decreases in the plasma membrane fraction of stimulated cells (Fig. 4). These results are qualitatively consistent with the internalization of fluorescent-CP visually observed by Niedel et al [20], who found patch formation at the cell surface within 2 min and internalization within 5 min. The 75% drop in specific CP-binding activity of the peak plasma membrane fraction approximates the magnitude of receptor "down-regulation" measured by Sullivan and Zigmond [4]. The results also extend the analysis of redistribution of CP binding activity measured in rabbit microsomal and granule fractions by Vitkauskas et al [21] by localizing these changes to the plasma membrane and rapidly sedimenting granule fractions. Of interest is the relatively unchanged amount of CP-binding activity in the gradient region containing specific granules and rapidly sedimenting, high-density galactosyl transferase activities [9]. This lends additional support to the evidence [3,7,8] that this fraction is not derived from plasma membrane and thus may represent, at least partially, an internal latent pool of activity which is either at a constant steady state level or acts as a reservoir of spare receptors.

The redistribution of CP-binding activity in the subcellular fractions after granulocyte stimulation by the same ligand can arise from a change in the binding

characteristics of the receptor, from its degradation, or from its redistribution. In this study we have shown that the receptor is probably the carrier of the ligand in the internalization process. In parallel studies [9,22] using N-formyl-Met-Leu-[^{3}H]Phe and a fluorescein derivative of N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys, we have measured the specific internalization rates of the formyl peptide in human granulocytes. If we assume that specific ligand (ie, receptor) internalization is directly proportional to receptor occupancy [2], then the maximum rate of specific receptor endocytosis would be about 8,000–10,000 receptor sites per min. There is also an approximate 1-min delay in internalization of ligand [9,22]. Therefore, in 5 min at saturating concentrations of N-formyl-Met-Leu-Phe, the cells should internalize 30,000 to 40,000 receptors or 60–80% of the original number expressed at the cell surface (50,000). The magnitude of this internalization matches the net loss observed in the plasma membrane fraction (75%). We therefore conclude that receptor down-regulation is primarily due to receptor internalization.

Intracellular Fate of the N-Formyl-Chemotactic Peptide Receptor: Binding Studies

We have assumed that all the receptor-binding activity in the rapidly sedimenting granule fraction from unstimulated cells is derived from overlapping plasma membrane (Fig. 4). This activity should therefore be 75% lower after stimulation. Since the specific activity of the binding remains the same and is observed as a new peak, we surmise that new receptor activity is being expressed which may have arisen by receptor redistribution after stimulation ($\sim$20% of that lost in the plasma membrane).

This redistribution could alternatively occur after fractionation as bulk plasma membrane aggregation with granules, presumably because cells normally agglutinate (within 2 min) under stimulatory conditions. Two pieces of evidence argue against this possibility. The first is that ^{125}I-WGA redistribution does not parallel that of receptor-binding activity (Table I). The other evidence is that stimulated (and agglutinated) cells which were prelabeled with NPH ^{125}I-peptide do not show significant levels of radioactivity (above control) in the dense granule fractions if the cells were cooled before internalization could take place (2 min). This evidence supports the hypothesis that the measured redistribution takes place by a specific cellular process.

The major source of the loss in receptor-binding activity in gradient fractions of stimulated cells can be explained by the internalization of receptor-ligand complex. We have already shown [9] that the tritiated form of N-formyl-Met-Leu-Phe can be incorporated into fractions containing low-density galactosyl transferase activity to an approximate level of 16,000 molecules/cell. In addition, this ligand is complexed in some relatively nondissociable form, stable after freezing and thawing, sonication, or even solubilization in detergent. The evidence presented in this paper suggests that receptor is the carrier of the peptide in this fraction and would, therefore, account for approximately 30% of the original number of receptors (50,000) on the unstimulated cell surface (40% of the activity down-regulated). These high-affinity receptors, therefore, would not be detectable in the peptide-binding assay because of their occupancy. The high affinity would thus explain the lack of any detectable increase in binding activity in sucrose density gradient regions corresponding to the low-density Golgi-enriched fractions. Thus, combining the binding activity appearing in the azurophil granule-rich fractions and that "sequestered" in the low-density galactosyl transferase fraction, we estimate that approximately 30% of the initial receptor-binding activity remains unaccounted for and may have been degraded.

Internal "Traffic" of Receptor in Subcellular Compartments

Recently, receptor recycling through internalization and reexpression at the cell surface has been postulated as a mechanism for receptor renewal during prolonged chemotactic migration of leukocytes [3,6]. Recycling has also been demonstrated or at least postulated for a number of ligand-receptor systems including insulin [23] and transferrin [24] as well as for other surface components of cells [25,26]. The evidence using the covalently labeled formyl-chemotactic peptide receptor indicates that occupied or labeled receptor does not leave the Golgi-enriched fraction even after 40 min of incubation of the cells at 37°C. This is in direct contrast to the transient residency ($t_{1/2}$ = 2–4 min) of *non*covalently attached and internalized formyl peptide in this fraction [9]. The persistence of the covalently coupled ligand receptor complex in this fraction can be explained by three possibilities: (1) receptor transfer to other compartments requires dissociation of the peptide-receptor complex; (2) the Golgi-enriched fraction is an endocytotic cul-de-sac for the receptor (ie, the receptor and ligand dissociate and the receptor remains in this fraction); or (3) the receptor structure has been altered in such a way as to inhibit intracellular transfer but not internalization.

The third possibility cannot be directly addressed at this time and must await adequate methods of receptor purification and structural analysis. The second possibility seems unlikely because we have not been able to detect any receptor-binding activity paralleling the distribution of internalized peptide and low-density galactosyl transferase activity even though the cell can accumulate peptide to at least three times the total number of surface receptor expressed [4,5]. The most interesting possibility is that peptide-receptor dissociation is required for receptor transfer out of the Golgi-enriched fraction. This possibility is supported by the observation that receptor binding activity and ^{125}I-WGA surface label appear to be redistributed to the dense granule fractions after stimulation.

Although there is insufficient evidence to prove that the receptor follows a particular pathway for recycling, taken as a whole our results are consistent with a receptor-flow pattern having aspects similar to the ones observed in iron transport [24], prolactin endocytosis [27], and general receptor-mediated endocytosis as summarized by Pastan and Willingham [28]. Peptide binds to the plasma membrane, is converted into a high-affinity form [9] which occurs either before or during the formation of surface clusters [29]. The clusters or patches are then internalized by a receptor-mediated endocytotic mechanism into "receptosomes" [28,29] or receptor mediated pinocytotic vesicles, (RMPV) [24]. These vesicles are transported to the Golgi or directly to dense granules or lysosomes where they may fuse. During the transfer or once in the granule, the peptide-receptor complex dissociates, freeing the peptide and allowing it to be released into the cytoplasm. The receptor, having been freed, can then shuttle back to the plasma membrane directly or via an internal latent pool from which it could travel the cycle again. Investigations are currently underway in our laboratory which should test the validity of such a scheme.

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