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Photoaffinity Labeling of the N-Formyl Peptide Receptor of Human Polymorphonuclear Leukocytes

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Quantitative analysis of ligand-occupied receptor interactions with elements of the cytoskeleton and with intracellular compartments requires a sensitive and simple method of identifying the receptor-ligand complex in living cells. Toward this goal, we have prepared a photoactivatable arylazide derivative of the chemotactic peptide N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys, which can be radiolabeled to high specific activity with ^{125}I. This derivative was biologically active as judged by its ability to elicit superoxide anion production by human PMNL at nanomolar concentrations (ED_{50} ~ 0.7 nM). When incubated at 0°C with whole PMNL, radioactive ligand became specifically and saturably associated with a 60–70,000-dalton species (as assessed by SDS-PAGE) after exposure to UV light. Addition of 10–100-fold excess of unlabeled parent or unlabeled azidopeptide derivative completely blocked uptake into this species. Approximately 20–40% of the available surface receptor-binding sites were covalently labeled under these conditions. Subcellular fractionation of the labeled cells on sucrose gradients after homogenization showed that the labeled species was primarily associated with plasma membrane-rich fractions. The labeled receptor could be completely solubilized with Triton X-100 in a form which eluted as a single species with a Stoke's radius of less than 50 Å on Sepharose 4B columns. In addition, the solubilized receptor-ligand complex bound specifically to wheat germ agglutinin, indicating that it is probably a glycoprotein. The ability to label the receptor in living PMNL with a high efficiency should facilitate the study of receptor dynamics and receptor physiochemical properties in this system.

Key words: N-formyl peptide receptor, photoaffinity labeling, polymorphonuclear leukocytes

Abbreviations used: PMNL, polymorphonuclear leukocytes; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide electrophoresis; DFP, diisopropylfluorophosphate; WGA, wheat germ agglutinin; NPH, 2-nitro-4-azidophenylaminohexanoyl; EDTA, ethylenediaminetetraacetic acid; TBS, 30 mM Tris HCl-0.13 M NaCl, pH 7.4; TCA, trichloroacetic acid; ED_{50}, effective dose yielding 50% of maximal biological activity.

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Polymorphonuclear leukocytes (PMNLs) possess specific receptors for a variety of chemotactic substances including C5a and bacterial-derived N-formylated peptides [1,2]. The release of such substances from sites of infection or inflammation plays a principal role in attracting PMNLs and other phagocytic cells to such sites [3]. Upon arrival these chemotactic substances also can cause these cells to release numerous toxic agents including superoxide anion (O_2^-) that are bactericidal and which can cause tissue damage [4].

Synthetic N-formylated peptides have been prepared [5], and numerous laboratories have shown that PMNLs have about 50–80,000 surface receptors per cell [6,7]. Niedel et al [7] have prepared a particularly useful derivative, N-Formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-COOH, that can be radioiodinated to very high specific activity and to which photoreactive reagents [8] or fluorescent reporter groups [9,10] can be attached via the free ϵ-amino group of the C-terminal lysyl residue.

Niedel and co-workers have recently shown that the above peptide can be specifically cross-linked to a 50–70,000-dalton polypeptide when incubated with PMNL plasma membrane preparations or detergent extracts in the presence of bifunctional amino cross-linking reagents as determined by SDS-PAGE [8,11]. Because the bifunctional cross-linking reagents are cytotoxic, these same workers prepared a photactivatable nitroazido derivative of the peptide that could also photolabel the same polypeptide with the hope that the receptor-ligand complex could be labeled in viable PMNLs. Unfortunately, it was found that even when using purified plasma membranes this particular derivative covalently labeled less than 0.1% of the total available cell-surface receptors.

In order to study receptor-ligand dynamics in this system, a need exists for a reagent that can covalently label the N-formyl peptide (NFP) receptor with high selectivity, high efficiency, and under conditions that do not impair cell viability so that cellular processing of the receptor-ligand can be determined. In this paper we report the synthesis and biological properties of a nitroazido derivative of NFP with a "spacer arm" between the peptide and the nitroazidophenyl group that meets all of the above criteria.

MATERIALS AND METHODS

Materials

CHO-Nle-Leu-Phe-Nle-Tyr-Lys was custom-synthesized by Bachem Laboratories (Torrance, California). The final product was pure as assessed by thin-layer chromatography and amino acid analysis. N-hydroxy-succinimidyl-6-(4′-azido-2′-nitrophenylamino) hexanoate was obtained from Pierce Chemical Co (Rockford, Illinois). N-formyl-Met-Leu-Phe, cytochrome c (type VI, horse heart), diisopropyl-fluorophosphate (DFP), wheat germ agglutinin (WGA), and Triton X-100 were obtained from Sigma (St Louis, Missouri). Carrier-free Na ^{125}I (505 mCi/ml) was obtained from Amersham (Arlington Heights, Illinois). Trasylol was purchased from Mobray Chemical Corp (New York). Urea, ultrapure, was from Schwarz/Mann (Spring Valley, New York). t-Boc-Phe-Leu-Phe-Leu-Phe was purchased from Vega Biochemicals (Tucson, Arizona). Rabbit antiserum against WGA was prepared by immunizing New Zealand rabbits with WGA using conventional immunization procedures.

Methods

Preparation of human polymorphonuclear leukocytes (PMNLs). Human cells were obtained from freshly drawn venous blood obtained from normal donors using acid citrate dextrose as an anticoagulant. PMNLs were isolated by the procedure of Henson and Oades [12] and resuspended in incubation buffer [10] at pH 7.4 (5 mM KCl, 147 mM NaCl, 1.9 mM KH_2PO_4, 1.1 mM Na_2HPO_4, 5.5 mM D-glucose, 1.5 mM $CaCl_2$, 0.3 mM $MgSO_4$, 1 mM $MgCl_2$) at a concentration of 5×10^7 cells/ml. No protein additives were used. The cell preparations were routinely composed of greater than 95% PMNLs, and viability as assessed by trypan blue exclusion was better than 95%. Cells were held on ice until used and remained fully responsive for up to 3–4 hr.

Preparation of photoactivatable N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys derivative. N-formyl-Nle-Leu-Phe-Nle-Tyr-N^{ϵ}-6-(4′-azido-2′-nitrophenylamino) hexanoyl)-Lys (NPH-peptide) was prepared by reacting the parent peptide (9.6 μmol) with N-hydroxysuccinimidyl-6-(4′-azido-2′-nitrophenylamino) hexanoate (19.2 μmol) in the presence of 4 ml of the dry dimethylformamide containing 20 μmol triethylamine for 16 hr at 22°C in the dark. The orange-red product was precipitated and washed with 10 vol of cold water and the pellet collected after it had been centrifuged and lyophylyzed. The residue was dissolved in dimethyl sulfoxide and purified by preparative thin-layer chromatography on a 2-mm thick silica gel plate developed with chloroform/methanol/triethylamine (5:2:1). Two major orange zones were seen, one at the solvent front, corresponding to free N-hydroxysuccinimidyl-6-(4′-azido-2′-nitro phenyl-amino) hexanoate, and one at $R_f = 0.64$ that was biologically active and nihydrin-negative. Underivatized peptide, which has an R_f of 0.2 in this solvent system, was not detected, indicating that the reaction was complete. The final material ($R_f = 0.64$) was eluted with dimethyl sulfoxide and stored in the dark at −20°C. The NPH-peptide was radioiodinated with Na ^{125}I by the chloramine-T method as described by Niedel et al [7] for the underivatized peptide. Final specific activity was 1,040 Ci/mmole.

Photoaffinity labeling of human PMNLs. PMNLs (3×10^6 cells/ml incubation buffer) and the indicated concentrations of NPH-^{125}I-peptide were reacted for 15 min on ice in glass test tubes under N_2 in the dark. Nonsaturable cross-linking was assessed in the presence of unlabeled peptides or peptide derivatives at concentrations indicated in the text. The cells were irradiated for 2 min on ice in a Rayonet UV light reactor (The Southern N.E. Ultraviolet Co, Middletown, Connecticut) at 360 nm. Preliminary experiments showed that this length of irradiation gave maximum covalent incorporation. After centrifuging (4°C, 10 min, 250g), the unwashed cell pellet was assessed for covalent labeling by SDS-PAGE or TCA precipitation (see below).

Subcellular fractionation of PMNL homogenates. PMNLs, labeled with NPH-^{125}I-peptide in the intact state, were homogenized by nitrogen cavitation and fractionated on linear isopycnic sucrose gradients as described by Jesaitis et al [13]. Briefly, $1-5 \times 10^7$ NPH-^{125}I-peptide-labeled cells/ml were washed twice with incubation buffer, resuspended in homogenization buffer (0.34 M sucrose, 10 mM Hepes, 0.1 mM MgC12, 1 mM EDTA, 1% Trasylol, pH 7.4) and then homogenized by N_2 cavitation after being equilibrated for 15 min at 400 psi at 4°C in a cell-disruption bomb (Parr Instruments, Moline, Illinois). The cell homogenate was centrifuged at 1,000g (5 min, 4°C) and the supernatant fraction was then layered onto sucrose

gradients (20–55%) and ultracentrifuged for 3 hr at 4°C in a Beckman L8-55 ultracentrifuge (Beckman Instruments, Palo Alto, California) at 83,000g. Two-milliliter fractions were collected and assayed for TCA-precipitable ^{125}I as described by Nordeen et al [14]. Markers for various subcellular organelles were as follows: plasma membrane, ^{125}I-WGA bound in the trace amounts to intact cells [15]; Golgi, UDP-galactosyltransferase [16]; specific granules and azurophil granules, lysozyme [17] and β-glucuronidase [18], respectively; and cytosol, lactic dehydrogenase [19].

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially according to Laemmli [20] in a minigel slab (1.5 mm) system [21] with the modification that in some experiments 8 M urea was incorporated into the gel matrix as indicated in the text. Fifty microliters of protein sample or a cell pellet of $< 3 \times 10^6$ of PMNLs was lysed with 100 μl (100°C) of a solution of 1 g urea plus 500 μl of 10% SDS containing 2 mM DFP, 5 mM EDTA, pH 7.2, and heated for 3 min at 100°C. Subsequently, 100 μl of 0.25 M Tris/glycine, pH 6.8, containing 25% 2-mercaptoethanol and 0.01% bromophenol blue as tracking dye was added and then heated for another 3 min to 100°C. The entire cell lysate was centrifuged and then the supernate was layered on the gel and electrophoresis was carried out at room temperature at 15 mA/slab for about 1 hr. Gels were fixed and stained in 25% 2-propanol, 10% acetic acid, 0.05% Coomassie brilliant blue G250, and destained in 10% acetic acid. Gels were dried on filter paper under vacuum and then exposed to X-OMAT AR film (Eastman Kodak Company, Rochester, New York) for from 1 hr to 3 days. In some instances a Cronet H1 PLUS YE enhancing screen (DuPont, Wilmington, Delaware) was used. Molecular weight standards were phosphorylase b (M_r = 43,000), bovine serum albumin (M_r = 67,000), ovalbumin (M_r = 43,000), carbonic anhydrase (M_r = 30,000), soybean trypsin inhibitor (M_r = 20,000), and α-lactalbumin (M_r = 14,400) and were purchased from Pharmacia Fine Chemicals (Piscataway, New Jersey).

Superoxide (O_2^-) anion assay. Production of O_2^- by PMNL suspension was performed by following the rate cytochrome c reduction in a continuous spectrophotometric assay as previously described [22,10]. Results were expressed as the maximal linear rate of O_2^- (nmol) produced per min per 10^6 cells.

Wheat germ agglutinin (WGA) immunoprecipitation of NPH-^{125}I-peptide receptor complex. PMNLs (2×10^8 cells) were photolabeled with NPH-^{125}I-peptide (5 nM) in the presence or absence of 50 nM nonradioactive peptide at 0°C as described above. After washing with incubation buffer by centrifugation (250g, 10 min), the respective cell pellets were frozen and thawed once and 1 ml of incubation buffer containing 5 mM EDTA, 2 mM DFP, and 1% (v/v) Triton X-100 added to each. After 20 min on ice, the Triton lysates were ultracentrifuged for 1 hr at 45,000 rpm at 5°C in a Beckman Ti50 rotor.

The resultant supernatant which contained better than 90% of the total TCA-precipitable specific radioactivity was used for subsequent immunoprecipitation studies as follows: 25 μl of WGA (20 mg/ml) was added to each extract (350 μl) and allowed to incubate for 30 min at room temperature followed by incubation overnight at 0°C. The next day 110 μl of a rabbit antiserum to WGA was added and incubated overnight in order to precipitate the added WGA. After centrifugation, the pellet was washed successively with incubation buffer and with 0.25 M sucrose-10 mM Hepes, pH 7.4, and radioactivity determined. The radioactivity which was elutable with N-acetyl-D-glucosamine, a specific saccharide inhibitor of WGA, was determined by incubating the pellets overnight in 0.2 M N-acetyl-D-glucosamine dissolved in 30

mM Tris-HCl-0.13 M NaCl (TBS) containing 0.1% NP-40, 1 mM $MgCl_2$, and 1 mM $CaCl_2$.

RESULTS

Biological Activity of NPH-Peptide

As shown in Table I, NPH-peptide was capable of eliciting the production of superoxide anion with an apparent ED_{50} of ~0.7 nM as compared to the parent peptide (ED_{50} ~ 2 nM). The biological activity of the derivative was not significantly affected by prior exposure of the ligand to an amount of UV irradiation sufficient to completely photolyze the nitroazidophenyl group (as determined by loss of absorbancy at 490 nm).

The biological activity was clearly mediated via the N-formyl peptide receptor because t-Boc-Phe-Leu-Phe-Leu-Phe (1 μM), a specific biological antagonist of the N-formylated peptides [23], completely blocked the observed biological response.

Covalent Labeling of the N-Formyl Peptide Receptor of Viable PMNLs

Human PMNLs were pretreated with 2 mM DFP, which inhibits proteases in this cell type [24], and after washing were incubated for 30 min at ice bath temperatures in the dark with NPH-^{125}I-peptide (2 nM) in the presence or absence of 50 nM unlabeled parent peptide. Under these conditions binding equilibrium was achieved and about 80% of all available surface receptors were occupied by ligand, assuming the K_D of the receptor for NPH-peptide is equivalent to its ED_{50} of 0.7 nM. The suspension was then exposed to UV irradiation (360 nm) for 2 min at 0°C. Preliminary experiments showed that 0.5–1-min exposure under the conditions employed was sufficient for maximal labeling. Without washing, the cells were then boiled with the presence of SDS sample buffer containing 2 mM DFP and subjected to SDS-PAGE. As shown in Figure 1 a major radioactive labeled species migrates with an apparent M_r of 60–70,000. Figure 1 also shows that the observed labeling was completely abolished if unlabeled parent peptide was present or if the photolysis step was omitted from the protocol (Fig. 1, lanes 2 and 3, respectively). In addition, unlabeled t-Boc-Phe-Leu-Phe-Leu-Phe (1 μM) and N-formyl-Met-Leu-Phe (50 nM) also inhibited labeling of the 60–70,000 dalton species (data not shown).

We noticed that a substantial proportion of the radioactivity remained at the top of the separation gel. This label appeared to be specific since it was abolished in the

TABLE I. Biological Response of Human PMNL to NPH-Peptide as Measured by O_2^- Production

Peptide derivative	Initial maximal linear rate of O_2^- produced (nmol/min – 10^6 PMNL)	ED_{50}[a] (nM)
NPH-peptide, unphotolysed	32	0.7
photolysed	30	1.5
Parent peptide	27	3.0
NPH-peptide, unphotolysed plus t-BOC-Phe-Leu-Phe-Leu-Phe (1 μM)	0	ND[b]
No peptide (buffer)	0	—

[a]ED_{50} = dose that produced a half-maximal response.
[b]ND = not done.

presence of excess unlabeled peptide (Fig. 1). Further investigation revealed that the material at the top of the gel was, in fact, aggregates composed of the 60,000-dalton monomer. We found that by treating the cells with 4% SDS and 8 M urea, followed by electrophoresis in a SDS-urea system, virtually all the TCA-precipitable radioactive label was present in the 60,000-dalton form (Fig. 2). Even with this gel system it was necessary to always use freshly prepared material and to avoid repeated freezing and thawing of the sample.

Concentration Dependence of the Labeling Reaction

We next wished to determine if the labeling reaction had a ligand concentration dependence that was comparable to the biological dose response. PMNLs were labeled at 0°C with various initial concentrations of NPH-^{125}I-peptide, photolyzed for 2 min, lysed, and separated by gel electrophoresis. The region of the gel containing the 60,000-dalton radioactive band was excised and the radioactivity determined. The data, corrected for background, are shown in Figure 3. As can be seen, the labeling reaction was saturable with an apparent ED_{50} of ~6 nM. Little if any nonspecific labeling was seen in the presence of 100-fold excess labeled peptide. In addition, as little as a ten-fold molar excess of unlabeled peptide inhibited the labeling reaction by 85% (data not shown). Based on a value of 50,000 surface receptors for PMNL and

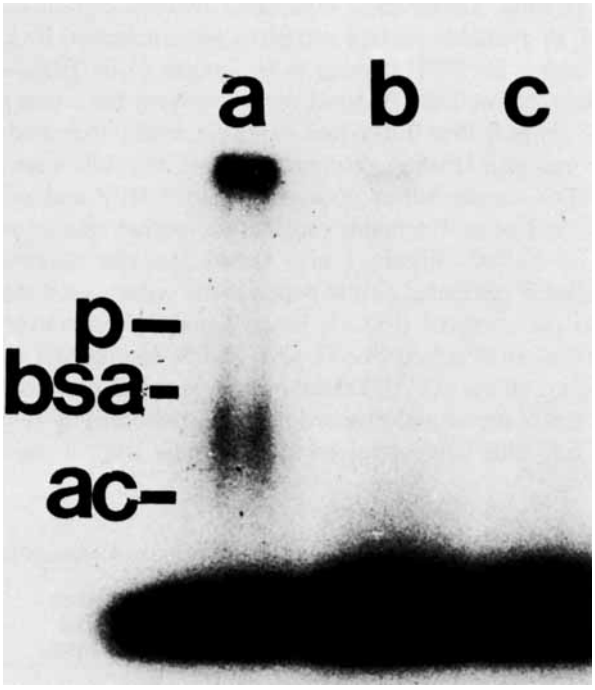

Fig. 1. Photoaffinity labeling of a 60,000-dalton species in intact PMNL by NPH ^{125}I-peptide. Autoradiogram of a 7% SDS polyacrylamide gel of SDS-extracted whole PMNL labeled with NPH-peptide (2 nM) for 30 min at 0°C. The cells were then exposed to UV light in the absence (lane a) or presence (lane b) of 40 nM nonradioactive underivatized parent peptide. As a control, cells were incubated as for lane a, except that exposure to UV light was omitted (lane c). Note the band that migrates between bovine serum albumin (bsa) and actin (ac). The position of phosphorylase b is designated by p.

a specific activity of 1,040 Ci/mmol for the ligand, the data indicate that about 40% of the total available surface receptors were covalently and specifically labeled by ligand under saturating conditions in this particular experiment. This value varies from 20–40% from one cell preparation to the next.

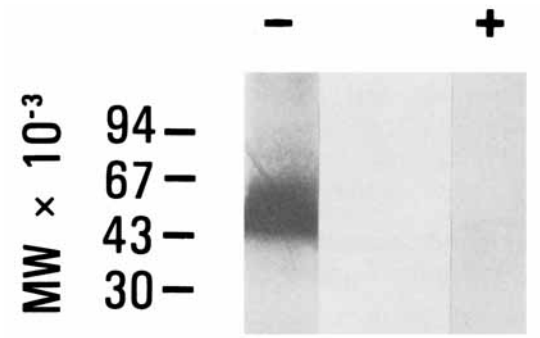

Fig. 2. An autoradiogram of a 9% polyacrylamide gel of NPH-^{125}I-peptide-labeled PMNL after electrophoresis in SDS-urea-PAGE. PMNL were labeled with NPH-^{125}I-peptide as in Figure 1, in the absence (−) or presence (+) of 100 nM nonradioactive parent peptide. In contrast to Figure 1, electrophoresis in the presence of SDS *and* urea (8 M) eliminated aggregation of labeled 60,000-dalton species seen at the top of the gel in the conventional Laemmli system. Note that the tracking dye had just run off the end of the separating gel.

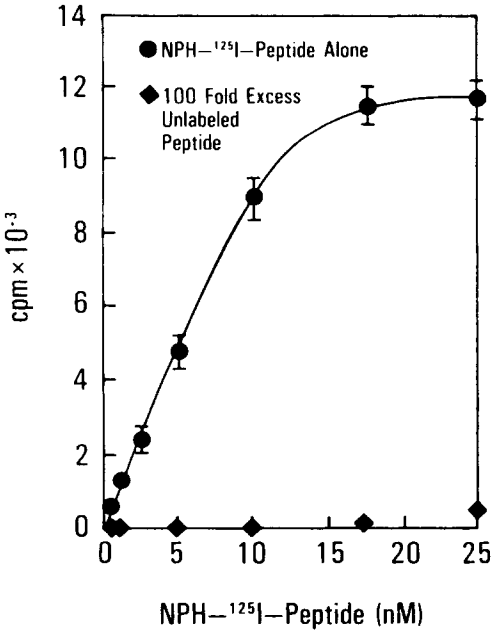

Fig. 3. Efficiency of photoaffinity labeling as a function of NPH-^{125}I-peptide concentration. Aliquots of 3 × 10^6 PMNLs/ml were incubated with the indicated concentrations of NPH-^{125}I-peptide in the absence (-•-) or presence (-◆-) of 100-fold excess of unlabeled parent hexapeptide for 15 min at 0°C in the dark. The cell suspensions were then irradiated at 360 nm for 2 min at 0°C and then subjected to SDS-urea-PAGE and assessed for covalently linked radioactivity as described in Figure 1.

Subcellular Localization of the TCA-Precipitable Radioactive Label After Homogenization of Labeled Cells

In order to determine if the cell-bound ligand was associated with the cell surface, PMNLs were labeled with NPH-^{125}I-peptide, washed, and lysed by N_2 cavitation. All procedures were performed at 0°C in order to minimize internalization of surface components. The 1,000g supernatant was then fractionated by isopycnic ultracentrifugation on a linear sucrose density gradient as described by Jesaitis et al [13]. As shown in Figure 4, the TCA-precipitable radioactivity was distributed predominantly in a single peak that cosedimented with plasma membrane (PM) markers but not with Golgi (GT), specific granule (SG), or azurophil granule (AG) marker enzymes. SDS-PAGE analysis confirmed that this label was associated with the 60,000-dalton species (data not shown).

Solubilization of the Ligand Labeled Receptor Complex and Its Identification as a Wheat Germ Agglutinin (WGA)-Binding Glycoprotein

Plasma membrane-rich fractions labeled with NPH-^{125}I-peptide were treated with 2% Triton X-100, 1 mM EDTA-TBS (pH 7.4) at 0°C. After ultracentrifugation for 2 hr at 100,000g, 90% of the TCA-precipitable label was found in the supernatant

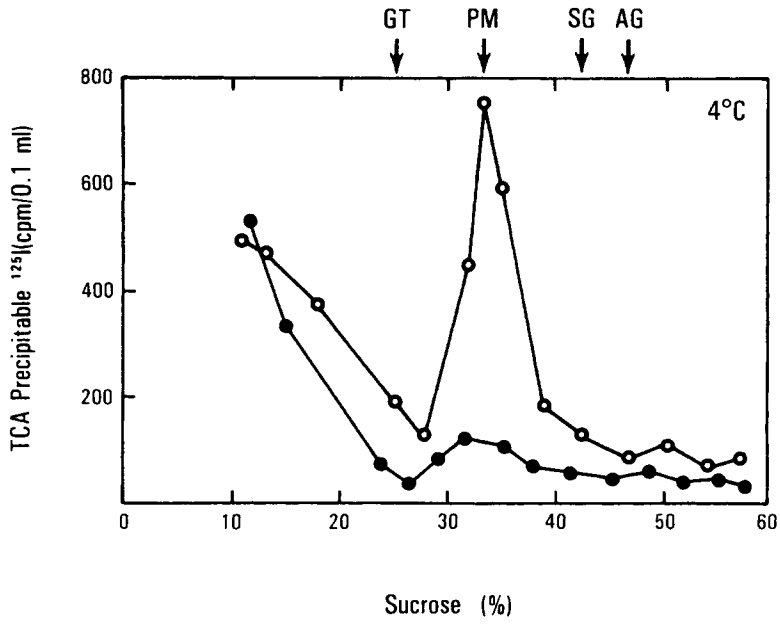

Fig. 4. Isopycnic sucrose gradient analysis of PMNL homogenates. 7×10^7 PMNLs were photoaffinity labeled with NPH-^{125}I-peptide (5 nM) at 4°C in the absence (○) or presence of 100 nM unlabeled parent peptide (●), washed at 4°C in incubation buffer + 0.1% bovine serum albumin, homogenized (N_2 cavitation) and subjected to isopycnic sucrose gradient centrifugation as described in the Materials and Methods section. NPH-^{125}I-peptide associated covalently with protein was measured by applying 0.1 ml (of 0.4 ml) of each fraction to a glass-fiber filter [13], washing the dried filter with cold 10% trichloroacetic acid, followed by acetone.

fraction, suggesting that the receptor-ligand complex was solubilized. In order to more rigorously show this, the Triton-EDTA extract was passed over a Sepharose column equilibrated with the same buffer. As shown in Figure 5, two peaks of radioactivity were seen. The larger one eluted with an apparent fractional elution volume of ~0.7; this species was largely absent if an excess of unlabeled peptide was included prior to the photolysis step and represented the specific receptor-associated label. This was subsequently confirmed by SDS-PAGE analysis (data not shown). The other peak, found in the internal column volume (V_i) was not abolished by addition of unlabeled peptide, was not TCA-precipitable, and therefore probably represented free radioactive ligand. Thus, by this criterion, the ligand-receptor complex is efficiently solubilized to a monodisperse form with a Stoke's radius of 50 Å or less.

It has been previously noted that wheat germ agglutinin (WGA) but not concanavalin A inhibits N-formyl-Met-Leu(^{3}H)-Phe binding to PMNLs [A. Jesaitis, unpublished results]. On the basis of this observation, a Triton extract of NPH-^{125}I-peptide-labeled PMNLs was immunoprecipitated. This was accomplished by treating the extract with WGA, followed by addition of an amount of anti-WGA antiserum sufficient to precipitate the added WGA. After extensive washing, the immunoprecipitate was found to contain 88.3% of the total specific TCA-precipitable radioactivity of the original extract (Table II). Furthermore, when this immunoprecipitate was treated with 0.2 M N-acetyl-D-glucosamine, 38% of the immunoprecipitable radioactivity was recovered in the supernatant after centrifugation. This was specific since 0.2 M sucrose had no such effect. SDS-PAGE analysis of the N-acetyl-D-glucosamine eluate showed that the major radioactive species migrated at with an apparent molecular weight of 60,000 daltons (data not shown).

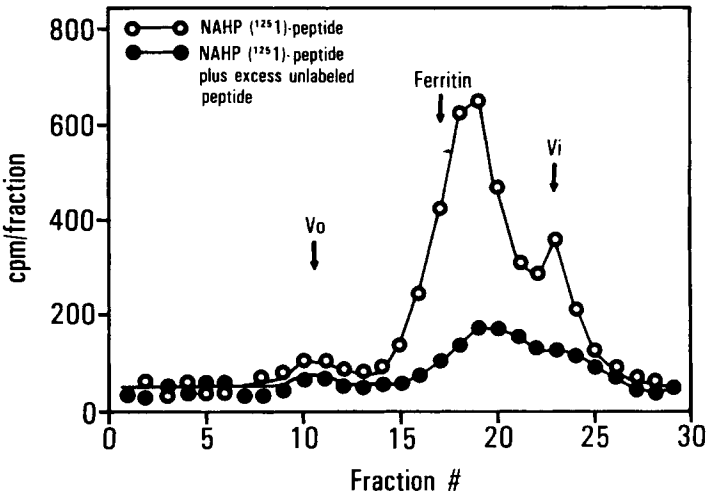

Fig. 5. Sepharose 4B chromatography of Triton X-100-solubilized plasma membranes isolated from PMNLs prelabeled with NPH-^{125}I-peptide at 0°C (as described in Fig. 4) in the *absence* (○) or *presence* (●) of nonradioactive peptide. Elution positions of the void volume (Vo), ferritin, and dinitrophenyl-L-lysine (Vi) are as indicated. The membranes were isolated as shown in Figure 4, pelleted by ultracentrifugation, dissolved in 2% Triton X-100-2mM EDTA in TBS, and applied to the column.

TABLE II. Immunoprecipitation of NPH-^{125}I-Peptide Receptor Complex From a Detergent Extract of PMNL by WGA and Anti-WGA*

Fraction	Total specific radioactivity recovered[a] (cpm)	Percent of total radioactivity recovered
Extract	36,000[b]	100
WGA + α-WGA precipitate	31,800	88.3
N-acetyl-D-glucosamine extract of precipitate	12,180	33.8

*See Materials and Methods for details.
[a]Corrected for radioactivity recovered from extracts of PMNL labeled with NPH-^{125}I-peptide in the presence of 50 nM nonradioactive peptide.
[b]TCA-precipitable radioactivity.

DISCUSSION

We report here the synthesis of a photoaffinity labeling reagent that meets all criteria as suitable for the study of the interaction of N-formyl peptide receptor in human PMNL with intracellular elements and a probe for studying the intracellular fate of the receptor-ligand complexes. In addition, we have demonstrated that this labeled receptor-ligand complex can be efficiently solubilized and subsequently partially isolated and characterized.

Previous reports have shown that NFP containing a lysine residue could be cross-linked to PMNL membranes by means of bifunctional imidates or other such reagents [8,10]. When analyzed on SDS-PAGE the specific label migrated with a M_r of 60,000 [8,10]. Niedel et al have previously reported the synthesis of a nitroazido derivative similar to ours with the exception that their compound lacked the hexanoate "spacer arm" between the arylazide ring and the N-formyl peptide ε-amino group of the C-terminal lysine residue [8]. These workers found that this compound labeled the receptor with very low efficiency (<0.1%, a result which we have likewise confirmed [M. Schmitt, unpublished observations]). When the hexanoyl "spacer arm" is added, efficiency of labeling was routinely high (20–40%) at saturating concentrations of ligand suggesting, as Niedel et al [8] anticipated, that the additional distance between the ligand binding site and the photoactive group may serve to bring the reactive end into close proximity with a reactive region of the receptor.

Our data also clearly show that a 60-kilodalton wheat germ agglutinin-binding protein is labeled by the NPH-peptide reagent with little if any nonspecific covalent labeling. Although these data do not rigorously prove that the labeled protein is a glycoprotein, they are strongly suggestive. In addition, the ease with which the protein aggregates upon storage in SDS and our recent finding of a closely clustered family (with respect to isoelectric points) of 60-kilodalton spots on two-dimensional O'Farrell [25] gels [26] all are consistent with the suggestion that the labeled species is a glycoprotein.

Several lines of evidence indicate that the labeled glycoprotein is in fact the receptor as operationally defined in the literature. First, the covalent labeling reaction shows a concentration dependence that is saturable at nanomolar concentrations (Fig. 3) and is similar to both the known K_D of 2 nM for the underivatized peptides and the ED_{50} with respect to superoxide production (Table I). Secondly, both the labeling reaction and the biological activity of NPH-peptide are completely abolished by the

competitive antagonist t-Boc-Phe-Leu-Phe-Leu-Phe [23]. Thirdly, we have recently shown that the covalently bound label, initially present in the plasma membrane-rich fraction (Fig. 4), is internalized by the PMNL at 37°C and is translocated to the Golgi-rich fractions in a manner that is kinetically and quantitatively indistinguishable from that seen with N-formyl-Met-Leu-[^{3}H] Phe [13]. This latter result is consistent with the notion that the NPH-peptide-labeled protein is the functional receptor and also shows that the covalently labeled species is processed in a manner that is similar to authentic N-formyl peptide. Thus, on the basis of both binding and functional criteria, the 60-kilodalton protein labeled by NPH-peptide appears to be closely associated with the ligand binding site and probably represents the binding unit of the biologically functional N-formyl peptide receptor.

Once labeled, the receptor-ligand complex can be quantitatively solubilized with a nonionic detergent such as Triton X100. The solubilized receptor is apparently a monodisperse component with respect to size and has a Stoke's radius of 50 Å or less (Fig. 5). On the basis of this preliminary data the solubilized receptor-ligand complex appears to have a Stoke's radius that is larger than might be expected for a globular molecule of 60,000 daltons. Whether this reflects a multisubunit or an asymmetric hydrodynamic shape is not yet known. Further studies are now underway with the goal of further characterizing the physical chemical properties of the receptor-ligand complex. The NPH-peptide described herein should greatly facilitate this work and allow elucidation of receptor interactions with other intracellular systems and compartments in living PMNL.

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