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# ISOLATION AND PARTIAL CHARACTERIZATION OF THE FORMYL PEPTIDE RECEPTOR COMPONENTS ON HUMAN NEUTROPHILS

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The receptor for formylated peptides such as FMLP has been reported to consist of glycoprotein components ranging from 24-95 kDa, and to exhibit both high and low affinity for ligand. Controversy exists on the molecular size and number of these components, and whether the different affinities represent distinct ligand binding sites. In this study, the receptor was found to be comprised of components, of 94, 68, and ≈40 kDa molecular size. Competitive binding inhibition experiments showed that FMLP bound to the components in the following order from highest to lowest affinity: 68 kDa > ≈40 kDa > 94 kDa. Our findings suggest that the FMLP receptor of human neutrophils contains at least three components, and that each component has a different affinity for FMLP. 

PMN chemotaxis can be stimulated by various N-formylated peptides such as FMLP, which have been thought to be structural analogs of bacterial products, and mimic the effect these products have on human PMN (1,2) by binding to specific PMN plasma membrane receptors (1,3) and causing an array of cellular responses in addition to chemotaxis (4-12). The interaction of FMLP with the membrane is associated with the activation of specific guanine nucleotides, via the action of regulatory guanine binding proteins (13, 14), which are involved in the receptor-mediated control of neutrophil function (15). Goetzl et.al. (16) isolated the FMLP receptor from human neutrophils, and found it to consist of several components, the major one having a molecular weight of 68 kDa. The chemotactic peptide bound primarily to this component. This was confirmed by the work of Niedel (17) and Marasco (18), who reported that the formyl peptide receptor on human PMN is of 50-70 kDa molecular weight. Allen et.al. (19) determined the receptor to be a single glycoprotein of approximately 63 kDa. The formyl peptide receptor has been reported to exist in two affinity states (20); stimulation of some neutrophil responses require much higher concentrations of formyl peptides than others (21-23). Controversy however exists as to whether these states represent different forms of a single receptor, or distinct membrane components capable of binding formylated chemotactic peptides with different affinities. Yuli et.al. (24) proposed that a particular cellular response is associated with a distinct high or low affinity receptors, and that either receptor can be regulated and, more specifically, favored by exogenous agents. On the other hand, Jesaitis et.al. (25, 26) suggested that a single receptor can be converted from a low affinity to a high affinity form, the latter form

## Abbreviations used

**BSA** = Bovine serum albumin; **CHAPS** = 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; **FMLP** = N-formyl-l-methionyl-l-leucyl-l-phenylalanine; **FMLP-lys** = N-formyl-l-methionyl-l-leucyl-l-phenylalanyl-l-lysine, acetate form; **PAL** = photoaffinity ligand (FMLP-lys-SASD); **PMN** = polymorphonuclear leukocytes; **PMSF** = phenylmethylsulfonyl fluoride; **R.T.** = room temperature; **SASD** = sulfosuccinimidyl 2-(p-azidosalicylamido) ethyl-1,3'- dithiopropionate; **SDS-PAGE** = sodium dodecyl sulfate polyacrylamide gel electrophoresis; **TLCK-HCl** = N-a-p-tosyl-l-lysine chloromethyl ketone hydrochloride.

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becoming inactive and internalized. Some investigators have even proposed that the observation of the different affinities may be due to poor control of experimental parameters (27). Feltner and Marasco (28) found three different receptor dissociation rates, and suggested that slowly dissociating receptors comprise two different receptor states, while the rapidly dissociating receptors may represent a third receptor state. In this study, we have isolated the FMLP receptor by affinity chromatography. Identity of the receptor from both column and membrane preparations was established using photoactivatable bifunctional labeling reagents. Competitive binding inhibition studies using different concentrations of chemotactic peptides showed that the components bind the ligand with different affinities. Our data suggests that: 1) the FMLP receptor in normal human PMN comprises at least three peptide components; 2) the major chemotactic peptide binding component is of 68 kDa molecular weight, while others are at 94 kDa, and ≈40 kDa; 3) these component appear to have different affinities for FMLP, in the following order from highest to lowest affinity: 68 kDa > ≈40 kDa > 94 kDa, supporting the idea that different affinity states of the receptor represent different ligand-binding sites.

## METHODS

**Isolation of Human Neutrophils**

Neutrophils were separated from buffy coats using the method described by De Nardin et.al. (29). The neutrophil yield from a typical 400 ml of donated blood was 1-3 x $10^9$ cells with a viability > 96%. Flow cytometric analysis using a Becton Dickinson FACScan showed this population to consist of >95% neutrophils.

**Purification of the FMLP receptor components**

Isolation of the FMLP receptor by affinity chromatography was accomplished using a modification of the method described by Goetzl (16). To obtain solubilized plasma membrane components, PMN were suspended (2 X $10^7$/ml) in a lysing buffer consisting of PBS, pH 7.2, containing 2.5 mM $MgCl_2$, 1 mg/ml ovalbumin, 1mM PMSF, 2 mM TLCK-HCl, and 20 µg/ml chymostatin, then processed at 4° C, as described (16). The final membrane preparation was then resuspended in chromatography buffer (0.02 M HEPES in 0.1 M NaCl, pH 7.2, containing 1% CHAPS). To detect potential proteolysis by PMN enzymes, [$^{14}$C]BSA (≈7 X $10^6$ CPM) was added to a sample aliquot of PMN, then subjected to the membrane preparation steps described above, at 4° C and at R.T. Integrity of the [$^{14}$C]BSA was checked by subjecting the first nitrogen cavitation lysate, and subsequent centrifugation supernatants to SDS-PAGE, followed by fluorography, using En$^3$Hance (New England Nuclear) to visualize radioactive bands. Four milliliters of the membrane preparation (0.25 mg of protein/ml) was then applied to the FMLP-agarose affinity matrix (16). After washing the column with several bed volumes of chromatography buffer, bound material was eluted with excess FMLP (10-60 µmol). Bound FMLP was removed from the receptor-containing fractions by dialysis against distilled $H_2O$, and removal was monitored by including a small amount of FML[$^3$H]P (New England Nuclear) in the eluting FMLP. The eluted material was then concentrated 25 fold using a Diaflo® ultrafiltration membrane (PM-10, molecular weight cutoff = 10,000, Amicon Corporation). To confirm the presence of the FMLP receptor, the eluate was subjected to photoaffinity labeling, followed by SDS-PAGE, and autoradiography.

**SDS-polyacrylamide gel electrophoresis**

SDS-PAGE was carried out according to the method of Laemmli (30). Separated protein components were visualized by silver staining (31).

**Conjugation of Photoaffinity Ligand**

A stable photoaffinity label (PAL) (32) was prepared by conjugating the acetate form of the tetrapeptide FMLP-lys to the photoreactive cross-linker SASD (Pierce Chemical Co.). Conjugation was carried out in aqueous medium following the method described by Wallenweber and Morrison (33). The resulting conjugate was stored in 100 µl aliquots at 4° C.

**Iodination of Conjugated PAL**

Radioiodination of the PAL preparation was carried out using a modification of the method described by Allen et.al. (1), and Shepard et.al. (34). Thirty micrograms of IODO-GEN (10 µg/100 µg protein, Pierce Chemical Co.) were dissolved in 100 µl chloroform, placed in an amber vial and dried to a thin film under nitrogen. One hundred microliters of the PAL preparation were then mixed in the IODO-GEN coated vial with 10 µl (1 mCi) of carrier-free Na[$^{125}$I] (Amersham International), and the reaction allowed to proceed for 30 mins. at R.T., with intermittent agitation. The sample was removed from the vessel and placed on a 1.3 x 27

cm Bio-Gel P-2 column (200-400 mesh), equilibrated with 20 mM NaOH, and eluted in the same buffer at a rate of 0.2 ml/min. Aliquots of 1.0 ml fractions were collected and monitored for radioactivity, then stored in the dark at 4° C.

**Photoaffinity Labeling**

Photoaffinity labeling of the membrane and receptor preparations was carried out at R.T. over 30 mins in the dark. One hundred micrograms of membrane or 20 µg of receptor proteins were treated with 1-2 nmol of [$^{125}$I]PAL (≈1.5 x $10^6$ cpm) in 20 µl 0.05 M Na borate buffer (pH 8). Covalent bonding of the label was achieved by photolysis for 10 min under UV light. The specificity of labeling was assessed by inhibiting the binding of the [$^{125}$I]PAL to the receptor preparation by pre-incubation with unlabeled excess FMLP-lys or PAL. The reaction mixtures were then analyzed by SDS-PAGE/autoradiography.

## RESULTS

**Characterization of the FMLP receptor.**

FMLP receptor components were affinity-purified from plasma membrane of normal PMN from different donors, pooled, concentrated by ultrafiltration, then analyzed by SDS-PAGE and amino acid analysis (Beckman System 6300 analyzer). The latter showed that approximately 1-1.5 mg of plasma membrane proteins and 10-25 µg of receptor proteins could be obtained from $10^9$ normal PMN. Elution of bound membrane components with ≤10 µmol of free FMLP resulted in the isolation of a 94 kDa component, as well as a 48 kDa component and a trace of a 68 kDa one (Fig. 1A, indicated by arrows); subsequent elution using higher amounts of free FMLP (≥60 µmol) resulted in the further elution of the 94 kDa component, as well as the 68 kDa component (Fig. 1B). Identical amounts of the non-formylated, non-chemotactic peptides Gly-Gly-Leu and Pro-Gly-Gly did not elute any detectable material from the affinity column. No

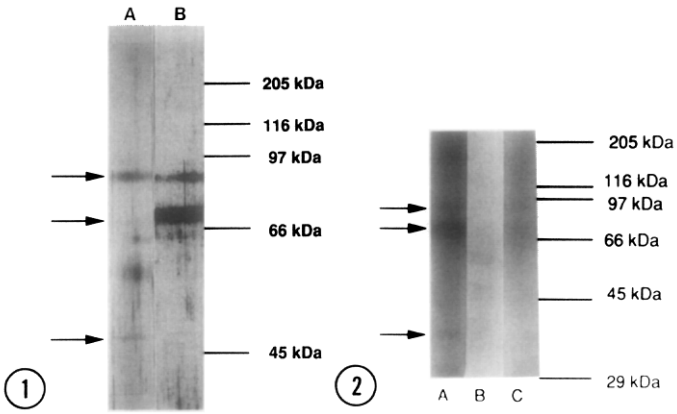


**Fig. 1. SDS-PAGE of affinity-purified FMLP receptor**

Concentrated FMLP receptor preparations were diluted 1:1 with sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% Glycerol) containing 10% (v/v) ß-mercaptoethanol as a reducing agent, heated at 100° C for 5 mins, then subjected to SDS-PAGE (7.5% acrylamide). The separated components were then visualized by silver staining. A = bound membrane components eluted with ≤10 µmol of free FMLP. B = bound membrane components eluted with ≥60 µmol of free FMLP.

**Fig. 2. Photoaffinity labeling of the FMLP receptor**

FMLP receptor from the FMLP-agarose column (≈0.5-1 µg protein/lane) was allowed to react with the [$^{125}$I]PAL, in the presence or absence of excess FMLP-lys, or excess non-radioiodinated PAL. It was then subjected to SDS-PAGE and autoradiography.

Lane A = FMLP receptor + [$^{125}$I]PAL.

Lane B = FMLP receptor + [$^{125}$I]PAL, in the presence of excess FMLP-lys.

Lane C = FMLP receptor + [$^{125}$I]PAL in the presence of excess non-radioiodinated PAL.

fragmentation of the [$^{14}$C]BSA proteolysis control was noted at either 4°C or at R.T., as such preparation appeared as a single band by itself as well as in all other steps tested.

All components from the receptor preparation were treated with the photoaffinity label ([$^{125}$I]PAL), then subjected to SDS-PAGE/autoradiography. Under the condition of these assays, the 68 and the 94 kDa component bound the radioiodinated chemotactic peptide. In addition, a ≈40 kDa component, which could not be detected by silver staining, was observed to bind the radioiodinated chemotactic peptide. These components were also observed when subjecting solubilized PMN membrane components to the photoaffinity labeling. In all cases, binding of the radioactive peptide could be inhibited by the pre-incubation with excess non-radioiodinated PAL or FMLP-lys before the photoactivation reaction (Fig. 2), but not with identical amounts of Gly-Gly-Leu and Pro-Gly-Gly.

**Competitive binding inhibition studies**

The observation that different amounts of FMLP were needed to elute the receptor components from affinity chromatography columns suggested the possibility that these components bound the ligand with different affinities. To test for this, solubilized PMN membranes were labeled with the [$^{125}$I]PAL (as described above) in the absence or presence of increasing concentrations of FMLP-lys. Samples were subjected to SDS-PAGE, then to autoradiography. The intensity of the labeled bands was quantitated using a LKB 2202 Ultroscan Laser Densitometer coupled to an Apple IIe microcomputer, by calculating the area under the curve of the peak generated by the band. Percent inhibition was determined by calculating the decrease in intensity of a band associated with a particular concentration of FMLP-lys when compared to the same sample labeled in the absence of FMLP-lys. Since the concentration of inhibiting ligand is relative to the concentration of the [$^{125}$I]PAL, results are expressed in relation to molar ratio of FMLP-lys to PAL, and are shown in Fig. 3. A 20 fold excess of FMLP-lys

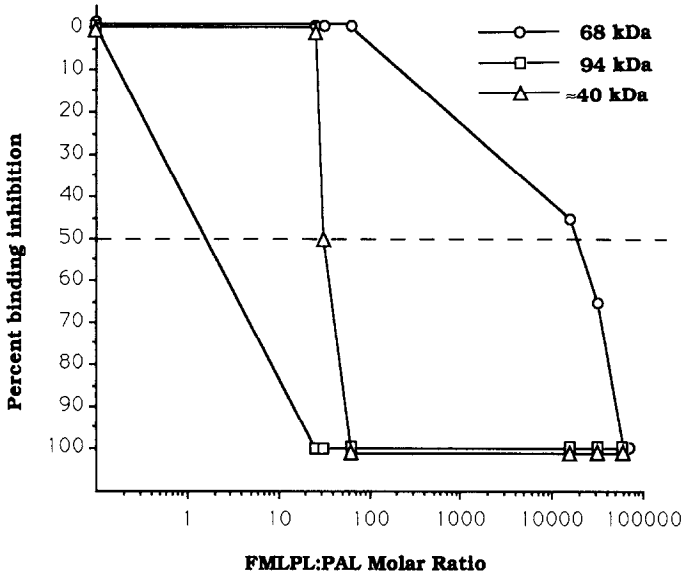


**Fig. 3. Inhibition of binding of PAL to solubilized membrane components by different concentrations of unlabeled FMLP-lys.**

Solubilized PMN membranes were labeled with the [$^{125}$I]PAL (as described above) in the absence or presence of increasing concentrations of FMLP-lys. Samples were subjected to SDS-PAGE, then to autoradiography. Percent inhibition was determined by calculating the decrease in intensity of a band associated with a particular concentration of FMLP-lys when compared to the same sample labeled in the absence of FMLP-lys.

with respect to the [$^{125}$I]PAL could completely inhibit the labeling of the 94 kDa component, while leaving the other components unaffected. A 35 fold excess of FMLP-lys was needed to inhibit the labeling of the ≈40 kDa component by about 50%, while complete inhibition was achieved at a FMLP-lys to [$^{125}$I]PAL molar ratio of 65. Inhibition of labeling the 68 kDa component could only be achieved at FMLP-lys to [$^{125}$I]PAL molar ratios above 15000, and maximal inhibition was achieved at a molar ratio of 64000. Identical amounts of the non-formylated, non-chemotactic peptides Gly-Gly-Leu and Pro-Gly-Gly had no effect on the binding of the [$^{125}$I]PAL to any of the three receptor components.

## DISCUSSION

In this study, we have isolated the receptor by affinity chromatography, and found it to be comprised of at least three, and possibly four components, of the following molecular size: 94kDa, 68 kDa, 48 kDa and ≈40 kDa. Only the 94, 68, and ≈40 kDa components could specifically bind a chemotactic peptide, with the 68 kDa moiety being the major ligand binding component. Furthermore, competitive binding inhibition studies demonstrated that each of the components capable of binding the peptide did so with different affinity; more specifically, the ranking of the different receptor components in order of descending affinity for the ligand was: 68 kDa > ≈40 kDa > 94 kDa. It is unlikely that the smaller components represent proteolytic fragments of the 68 or 94 kDa ones, since no proteolysis was detected in all membrane preparation procedures when using [$^{14}$C]BSA as a control. The FMLP receptor is a glycoprotein with two N-linked oligosaccharide chains. Sequential deglycosilation results in the reduction in the molecular weight to 40-45 kDa and finally to 33 kDa for the fully deglycosylated form (35). It is possible that the 48 and the ≈40 kDa components may reflect partially deglycosylated forms of the receptor. Indeed, Goetzl et.al. (16) had reported receptor components of similar molecular weight, but failed to demonstrate the binding capacity of the 94 kDa one. Since such component, as well as the others, was isolated by FMLP affinity chromatography, one must assume a good possibility that this component may specifically bind the ligand as the others do. The fact that the 94 kDa component was not shown to specifically bind a chemotactic peptide in Goetzl's study may have been due to the unavailability of techniques such as the use of photoaffinity reagents which are extremely sensitive tools for the detection of this binding. One question may arise as to why such enormous molar excesses of FMLP-lys are needed to displace the [$^{125}$I]PAL from the higher affinity receptor components while only a modest molar excess of the unlabeled PAL can achieve the same effect. This phenomenon has been reported before (36) and is mainly due to the fact that the PAL has a much higher biological activity and affinity for its receptor than the native ligand by itself. Recently, Murphy et.al. (37) have suggested that the FMLP receptor in HL-60 cells is encoded by a 2kb size class of mRNA, representing a single polypeptide chain. They arrived to such conclusion by injecting HL-60 RNA into Xenopus oocytes, and observing chemotactic peptide-stimulated responses in such cells. Since no FMLP receptor was demonstrated at the surface of the oocytes, one must wonder whether such cellular activities truly represent expression of FMLP receptors. Furthermore, the finding of a single 2kb mRNA encoding for the FMLP receptor is in contrast to the findings of Boulay et.al. (38, 39) who have isolated clones encoding for the FMLP receptor from a CDM8 expression library of differentiated HL-60 cells. These clones can transfer to COS-7 cells the capacity to specifically bind a chemotactic peptide, and can induce the expression of the FMLP receptor on the surface of these cells. Northern analysis demonstrated that these clones represent transcripts of 1.6, 1.9, 2.3, and 3.1 kb, suggesting the existence of related receptor components. Indeed, these investigators

have described the isolation of more than one receptor components, supporting our suggestion that the functional FMLP receptor may be made of multiple subunits.

In this study, we have isolated components of the PMN plasma membrane which specifically bind a formylated chemotactic peptide, confirming the work of Goetzl et.al. (16), and have demonstrated that each of these moieties binds such peptide with different affinity. Our findings suggest that the FMLP receptor complex is composed of more than one component, and that the different affinity states which have been described for the FMLP receptor may be associated with distinct ligand binding sites.

## ACKNOWLEDGMENTS

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