A Chemoattractant Receptor on Macrophages Exists in Two Affinity States Regulated by Guanine Nucleotides

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ABSTRACT The binding characteristics of the oligopeptide chemoattractant receptor on guinea pig macrophages and macrophage membrane preparations were characterized using detailed binding studies and computer analysis. Viable macrophages bound the radiolabeled chemoattractant *N*-formyl-methionyl-leucyl-[^{3}H]phenylalanine with single dissociation constant (K_D) of 18.4 ± 4.6 nM with 15,300 ± 1,800 sites per cell. Binding data from membrane preparations indicated the presence of two classes of binding sites with K_D of 1.5 ± 0.4 nM and 25.5 ± 11.0 nM. Approximately 23% of the receptors were in the high affinity state. In the presence of added guanine nucleotide di- or triphosphates, the high affinity receptors in the membrane preparations were converted to low affinity states with no change in the total receptor number. Nonhydrolyzable derivatives of GTP were most potent in converting the receptor from its high to low affinity state. These data suggest that the affinity state of the oligopeptide chemoattractant receptor in macrophages is regulated by guanine nucleotides and GTPase, implying that the transduction mechanisms of this receptor may be controlled by a guanine nucleotide regulatory unit.

Macrophages are motile cells capable of migrating along increasing gradients of chemoattractants. Higher concentrations of chemotactic factors than those required to initiate directed migration stimulate these cells to secrete lysosomal enzymes and produce superoxide anions (15). Several years ago we demonstrated that macrophage chemotaxis was initiated by the binding of chemoattractants to specific cell surface receptors (14). To understand how complex biological responses in macrophages are initiated by chemotactic stimuli, it will be important to delineate the binding characteristics of this receptor in detail and to identify its means of regulation. To this end we have developed methods for studying receptor-chemoattractant interactions in macrophage membrane preparations as well as in intact cells. Similar types of receptor analysis have been instrumental for characterizing the transduction mechanisms of other hormone-receptor systems, particularly those involving the neurotransmitters (20).

This manuscript describes studies of the binding of the chemoattractant *N*-formyl-methionyl-leucyl-[^{3}H]phenylalanine (FML[^{3}H]P)[1] to guinea pig peritoneal macrophages and membrane preparations thereof using a computer program, SCTFIT, to analyze binding data (5). The program allows rigorous statistical analysis of binding data and is particularly useful for the study of receptors that may exist in multiple affinities (3). These methods were also used to determine the role of nucleotides in the regulation of the macrophage chemotactic factor receptor.

MATERIALS AND METHODS

Chemicals: FML[^{3}H]P (specific activity 46.7 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Guanylyl-imidodiphosphate (p[NH]

[1] *Abbreviations used in this paper:* AMP, adenosine-5′-monophosphate; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; FML[^{3}H]P, *N*-formyl-methionyl-leucyl-[^{3}H]phenylalanine; GDP, guanosine 5′-diphosphate; GMP, guanosine-5′-monophosphate; GTP, guanosine-5′-triphosphate; GTPγS, guanosine-5′-0-3-thiotriphosphate; PMSF, phenylmethylsulfonyl fluoride; p[NH]ppG, guanylyl-imidodiphosphate.

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ppG) and guanosine-5′-0-3-thiotriphosphate (GTPγS) were obtained from Boehringer Mannheim (Mannheim, Federal Republic of Germany). Guanosine-5′-triphosphate (GTP), guanosine-5′-diphosphate (GDP), guanosine-5′-monophosphate (GMP), and adenosine-5′-monophosphate (AMP), shellfish glycogen, phenylmethylsulfonyl fluoride (PMSF), and *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Preparation: Inflammatory macrophages were obtained by peritoneal lavage of male Hartley (Camm Research, Wayne, NJ) guinea pigs, 400–600 g, 3 d after intraperitoneal injection with 30 ml of 0.75% (wt/vol) shellfish glycogen in isotonic saline (6). The cells were subjected to hypotonic lysis and the resulting cell populations contained ~90% macrophages. FML[^{3}H]P binding to intact cells was performed at cell concentrations of 1.5×10^6/ml in incubation buffer, (0.14 M NaCl, 1.9 mM KH_2PO_4, 5.1 mM Na_2HPO_4, 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.4) plus 1 mM PMSF. Nonspecific binding, which was ~6% of total binding in the presence of PMSF, was empirically found to be 50% greater in the absence of PMSF.

Membrane Preparation: Membranes were prepared by the method of Davies et al. (2). Macrophages were resuspended in 30 ml of ice-cold buffer (50 mM Tris, 10 mM $MgCl_2$, pH 7.7) and disrupted twice with a Tekmar polytron (Tekmar Co., Cincinnati, OH) for 20 s. This suspension was centrifuged at 200 *g* for 10 min at 4°C, the pellet discarded, and the supernatant centrifuged at 41,000 *g* for 10 min at 4°C. This pellet was washed twice by resuspending with a teflon pestle in 30 ml of Tris buffer (50 mM Tris, 10 mM $MgCl_2$, pH 7.4). Final membrane preparations contained ~0.5 mg protein/ml in Tris buffer, pH 7.4. 5′ nucleotidase levels in the membrane preparation were enhanced approximately 5.1-fold above the levels present in crude cell homogenates and 10-fold above the levels in the cytosolic fraction.

FML[^{3}H]P Binding Assay: Membranes or cells were incubated in a total volume of 0.15 ml buffer containing 0.25–40 nM FML[^{3}H]P in the presence and absence of 100 μM unlabeled FMLP (21). Incubations were at 25°C for 25 min (membranes) or 60 min (intact cells). The incubation times were chosen in accord with the requirements for binding to reach steady state. In experiments where nucleotide effects on binding were studied, membrane preparations were preincubated with the indicated compounds at 25°C for 20 min before addition to FML[^{3}H]P and were present during the entire incubation period. Binding was terminated by rapid vacuum filtration onto Titertek harvester (Flow Laboratories, McLean, VA) filters followed by washing with 11 ml of ice-cold buffer. Radioactivity on the filters was counted in an Intertechnique scintillation counter (IN/US Service Corp., Fairfield, NJ) with a 60% counting efficiency.

Computer Modeling: Data from binding isotherms of FML[^{3}H]P to intact cells and membranes was computer modeled using the method of Hancock et al. (5). The program, SCTFIT, is based on the principle of mass action ligand-receptor interactions and uses a nonlinear least squares curve fitting method (4). This model allows for analysis of receptors that may exist in multiple affinity states. All data were analyzed using a one- and two-site model. The two-site model was accepted only when the fit to the data was significantly improved ($P < 0.01$) on the basis of the lowest values of mean squares of residuals. Affinity constants and concentrations of each class of receptors represent the geometric means ± SE (3).

Intracellular Nucleotide Levels: Inflammatory macrophages were prepared as described above, pelleted, and frozen in liquid nitrogen. The pellet was extracted with 6% trichloroacetic acid and the supernatant neutralized with tri-*N*-octylamine in Freon. The aqueous phase was analyzed for nucleotide levels on a Whatman Partisil-10 SAX (Whatman, Inc., Clifton, NJ) anion exchange column using high-pressure liquid chromatography (12). The intracellular guanine nucleotide levels were: GMP <2.4 μM, GDP = 67 μM, GTP = 144 μM. The intracellular adenine nucleotide levels were AMP = 70 μM, ADP = 293 μM, ATP = 1 mM.

RESULTS

FML[^{3}H]P Binding to Intact Macrophages

Inflammatory macrophages contain on their surface specific saturable receptors for the oligopeptide chemotactic factor FML[^{3}H]P (14). To better characterize these receptors, we incubated various concentrations of FML[^{3}H]P with intact guinea pig macrophages and the binding isotherms were computer modeled. Fig. 1 illustrates one such binding isotherm, the data of which were demonstrable of a single class of receptor sites. The dissociation constant (K_D) was 24.6 nM with 17,800 receptors per cell. The K_D determined by Scatchard analysis was 29.5 nM with 16,900 receptors per cell,

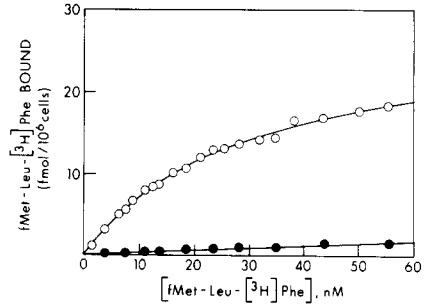

FIGURE 1 Binding isotherm of FML[^{3}H]P to intact macrophages. O, total ligand bound. ●, nonspecific binding defined as the amount of radioligand bound in the presence of 100 μM unlabeled ligand. The lines through the open and closed circles are the computer derived best fits for the total binding and nonspecific binding, respectively. A K_D of 24.6 nM and 17,800 receptors per cell were determined from this data.

values in good agreement with those derived by SCTFIT. In nine computer modeled experiments, intact macrophages were shown to have an average of 15,300 ± 1,000 receptors with a single affinity of 18.4 ± 4.6 nM.

FML[^{3}H]P Binding to Macrophage Membrane Preparations

Binding studies of FML[^{3}H]P to macrophage membrane preparations were also performed and Fig. 2 represents a typical binding isotherm. These data suggest heterogeneity of binding sites and are compatible with two populations of receptors with different affinity states. The dotted line represents the computer derived best fit to the data assuming a single class of receptors with one affinity state. The solid line is the best fit calculated from the same data assuming the receptor exists in two affinity states. Least squares analysis demonstrates that the two affinity models provides a statistically better fit for the data ($P < 0.001$). The affinities derived for the two sites were 1.6 nM and 38.0 nM respectively with 21% of the receptors representing the high affinity form. Averages from 10 computer modeled experiments demonstrated that 23.3 ± 0.1% of the receptors had an high affinity with a K_D = 1.5 ± 0.4 nM and the low affinity receptors had a K_D = 25.5 ± 11 nM. The K_D of the low affinity site in membranes is statistically indistinguishable from the single affinity observed in intact cell preparations. Scatchard analysis of this same data was curvilinear, as expected for a heterogeneous population of receptors. Assuming the curvilinear plot was due to two noninteracting receptor populations, two affinities can be estimated to be 2.1 and 19.1 nM with ~27% of the receptors in the high affinity state.

Effect of Guanine Nucleotides on FML[^{3}H]P Binding in Membranes

To study the effects of guanine nucleotides on FML[^{3}H]P binding, we preincubated membranes for 20 min at 25°C with the nonhydrolyzable derivative of GTP, p[NH]ppG, or with buffer alone. Fig. 3 shows that the binding of FML[^{3}H]P is decreased in the presence of 10^{-4} M p[NH]ppG. This decrease in binding appears to result from a conversion of high affinity receptors to low affinity receptors and not from a decrease in total receptor sites. Total binding in the presence of guanine nucleotide was diminished because the binding isotherms were carried out to 40 nM FML[^{3}H]P, a dose that occupies a maximum of ~60% of the low affinity sites. When the specific

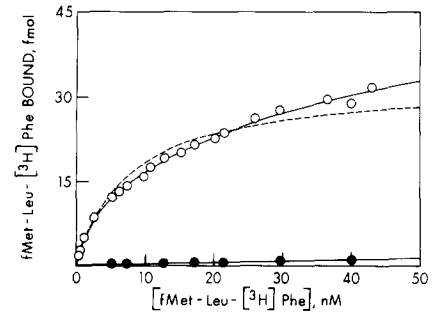

FIGURE 2 Binding isotherm of FML[^{3}H]P to macrophage membrane preparations. The dotted line represents a one site fit to the data while the solid line represents a two site fit to the same data. The fit derived from a two site model was significantly better ($P < 0.001$) than that derived from a one site model. The lower line (●) is nonspecific binding. The two affinities derived from this experiment are 1.6 and 38.0 nM with 21% high affinity sites.

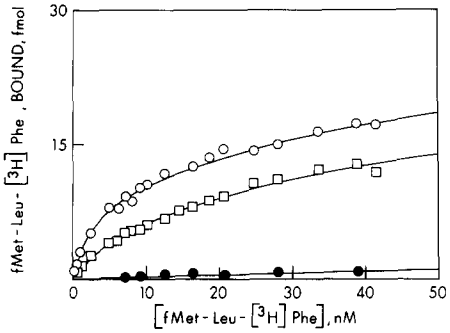

FIGURE 3 Binding isotherms of FML[^{3}H]P to macrophage membrane preparations in the presence and absence of p[NH]ppG. Membranes were preincubated for 20 min at 25°C with buffer alone or with 10^{-4} M p[NH]ppG. □, total binding in the presence of p[NH]ppG; ○, total binding in buffer alone. ●, nonspecific binding. The reduction in binding is due to a shift from 29.4% high affinity sites in buffer alone to 8.7% in the presence of p[NH]ppG.

binding data are extrapolated to complete saturation using SCTFIT, both treated and untreated membrane preparations had approximately the same number of receptors (treated: 0.133 ± 0.014 nM; untreated: 0.143 ± 0.020 nM). The total number of high affinity receptors was decreased from 29.4 to 8.7% with a concomitant increase in low affinity sites. In this, plus three other experiments, the average number of high affinity sites was reduced from 23.2 ± 0.2 to 2.3 ± 0.8%. There was no significant change in the K_D of the high (K_{DH}) or low affinity (K_{DL}) sites following incubation with p[NH]ppG (K_{DH} = 1.92 ± 0.86 vs. 1.03 ± 0.96; K_{DL} = 23.2 ± 12.49 vs. 30.0 ± 8.12, respectively).

Specificity of Guanine Nucleotide Effect on Binding

Other nucleotides were tested to determine the specificity of the p[NH]ppG effect on FML[^{3}H]P binding. Table I summarizes the results as the percent decrease in high affinity binding sites produced by each nucleotide tested as determined by analysis of detailed binding isotherms. The guanine nucleotide triphosphates p[NH]ppG, GTPγS, and GTP significantly decreased high affinity binding as did GDP to a lesser degree while GMP had no effect. Neither ATP, its nonhydrolyzable derivative p[NH]ppA nor CTP had any significant effect on binding. The guanine nucleotides inhibited FML[^{3}H]P binding in a dose response fashion as is indicated in Fig. 4. The nonhydrolyzable derivatives of GTP were the most potent inhibitors of FML[^{3}H]P binding followed by GTP. The EC_{50} for the nonhydrolyzable derivatives of GTP were ~5 × 10^{-6} M while the EC_{50} for GTP was 5 × 10^{-5} M. Neither ATP nor p[NH]ppA had an observable effect on binding at concentrations as high as 10^{-4} M.

TABLE I

Effect of Nucleotides on Expression of High Affinity Sites

Nucleotide added*	% Decrease of high affinity binding sites‡
None	—
p[NH]ppG	100
GTPγS	90.1
GTP	79.4
GDP	45.5
GMP	0
p[NH]ppA	11.6
ATP	0
CTP	15

* Membranes were preincubated for 20 min at 25°C with 10^{-4} M of the indicated nucleotide. Nucleotides were present during the binding assay.

‡ The indicated values represent the percent decrease of high affinity binding sites for each nucleotide as compared with the buffer control. The percent high affinity sites for each experimental condition was determined by computer analysis of 20 point binding isotherms. Each of the above determinations was repeated at least once with similar results.

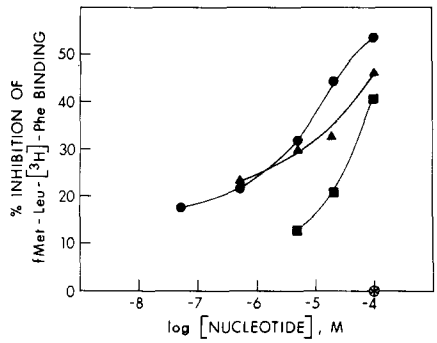

FIGURE 4 Dose response effects of nucleotides on FML[^{3}H]P binding to membrane preparations. The membranes were preincubated with the indicated concentration of nucleotide at 25°C for 20 min, then 8.5 nM FML[^{3}H]P was added. ●, p[NH]ppG. ▲, GTPγS. ■, GTP. *, ATP. ○, p[NH]ppA. The data is plotted as the % inhibition of specific binding.

DISCUSSION

Viable guinea pig macrophages contain specific receptors for oligopeptide chemotactic factors on their surfaces (14). To characterize the regulation of this receptor, we performed detailed binding studies of FML[^{3}H]P to intact cells and to macrophage membrane preparations. Analysis of data with intact macrophages indicates a homogenous class of receptors with a single affinity of 18 ± 4.6 nM and 15,300 ± 1,800 receptors per cell. These results are in agreement with those previously reported using conventional Scatchard analysis (14). Similar to intact cells, macrophage membranes bound FML[^{3}H]P in a specific and saturable fashion. The specificity of the membrane receptor for a series of N-formylated oligopeptides was identical to the receptor on intact macrophages (14). In contrast to intact cells however, membrane binding data was most consistent with a heterogeneous population of

receptors. Heterogeneous binding of the N-formylated peptides to human and rabbit polymorphonuclear leukocytes has also been recently reported (7, 9, 13). In the present study using macrophage membrane preparations, two classes of receptors were distinguished by two affinities with K_D of 1.5 ± 0.4 nM and 25.5 ± 11.0 nM, respectively. An average of 23.3 ± 0.1% of the receptors expressed the high affinity state. The single affinity observed in the intact macrophage is not statistically distinguishable from the lower affinity site in membranes.

Heterogeneous receptor sites have been demonstrated in other hormone-receptor systems. For example, in the adrenergic and cholinergic receptors, guanine nucleotides have been shown to regulate receptor binding and to be required for stimulus-response coupling (11, 17, 19). The effects of guanine nucleotides on FML[^{3}H]P binding to macrophage membranes were therefore examined. The data show that p[NH]ppG, when present during binding, produced a 90.1% reduction in the percentage of high affinity sites without a change in total receptor number. These data imply that p[NH]ppG prevents the conversion of low affinity to high affinity sites and/or converts high affinity sites to low affinity. Other guanine nucleotides tested GTP, GDP, and the nonhydrolyzable derivative GTPγS produced similar effects on binding while GMP, ATP, p[NH]ppA, and CTP had no effect. Inhibition of binding by the guanine nucleotides was dose dependent and greatest with nonhydrolyzable derivatives of GTP. These data suggest that at least a portion of the binding sites detectable in macrophage membranes are an interconvertible class of receptors and that a GTPase is involved in the regulation of receptor affinity. The possibility that the detection of high affinity binding sites in the membranes was due to a unique intracellular pool of receptors cannot be excluded since the membrane preparations used contained β-glucuronidase, a lysosomal marker. However, the convertibility of the high affinity to low affinity sites with guanine nucleotides indicates that the former are not a unique population of receptors. In addition, in experiments not shown, disrupted macrophages were fractionated on discontinuous sucrose density (10, 18) and Percoll density gradients (1). In all cases, specific FML[^{3}H]P binding was seen only in fractions containing the plasma membrane marker 5′nucleotidase. In none of the fractions however could plasma membranes be obtained free of β-glucuronidase activity.

The data presented here resemble certain characteristics of neurotransmitter receptors. In the β-adrenergic receptor a single affinity is observed in the intact cells while membranes display a second higher affinity that is converted to a low affinity form by guanine nucleotides (17). Transduction mechanisms of the β-adrenergic receptor have been shown to be mediated by a nucleotide regulatory protein (N-protein) (16). A ternary complex model similar to that proposed for the β-adrenergic system (17) is consistent with the FML[^{3}H]P binding data presented here and offers a possible explanation for the guanine nucleotide effect. Fig. 5 presents a functional model adapted for the chemotactic factor receptor on macrophages. In this model, receptor occupancy by a chemoattractant (CTX) facilitates the substitution of GDP by GTP on a nucleotide regulatory unit (N), thereby providing N-GTP to activate an effector (E → E*). The affinity of the receptor is determined allosterically via its binding to the N-unit. When coupled to the N-unit carrying GDP or GTP, the receptor expresses a low affinity state. We postulate that the high affinity state of the receptor (R′) is manifest when the receptor is either free of N or bound to N in the absence of any guanine nucleotide. Similar findings are present in human granulocyte membranes suggesting that the proposed model may be generally relevant to chemotactically responsive cells (8). Moreover, we have recently shown that guanine triphosphates are required for the activation of adenylate cyclase by prostaglandins in guinea pig macrophage membranes (19).

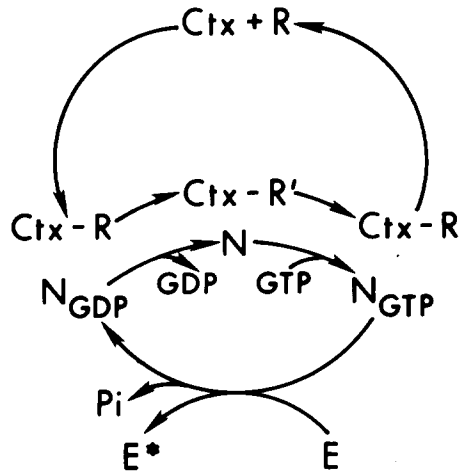

FIGURE 5 Postulated model for the interaction of guanine nucleotides with a nucleotide regulatory protein and the chemoattractant (*CTX*)-receptor complex. It is proposed that the affinity of the receptor is affected by its coupling to a regulatory unit, *N*, which binds guanine nucleotides. The transient high affinity state (*R′*) may occur when the receptor is bound to free N. (*E* and *E**) The unmodified and modified forms of the putative effector molecule.

The model of the chemoattractant receptor proposed here is consistent with the detection of only a single low affinity class of receptors in intact macrophages since the levels of guanine nucleotides in the cells are sufficient to allow receptor interconvertibility in situ and thus expression of one affinity. The levels of GTP and GDP in guinea pig macrophages were 114 and 67 μM, respectively. In membrane preparations however, when the guanine nucleotides are removed by washing, an additional population of receptors can be detected since interconvertibility is blocked. The effector(s) for the chemoattractant receptor is yet unknown but likely candidates would include a methyltransferase, a phospholipase, a protein kinase or perhaps adenylate cyclase. In any case, guanine nucleotide modulation of FML[^{3}H]P binding implies a mechanism for the regulation of stimulus-response coupling for chemoattractant receptors on macrophages.

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