Solubilization and Reconstitution of the Formylmethionylleucylphenylalanine Receptor Coupled to Guanine Nucleotide Regulatory Protein†

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ABSTRACT: We describe the solubilization, resolution, and reconstitution of the formylmethionylleucylphenylalanine (fMet-Leu-Phe) receptor and guanine nucleotide regulatory proteins (G-proteins). The receptor was solubilized with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. Guanine nucleotides decreased the number of high-affinity binding sites and accelerated the rate of dissociation of the receptor–ligand complex, suggesting that the solubilized receptor remained coupled to endogenous G-proteins. The solubilized receptor was resolved from endogenous G-proteins by fractionation on a wheat germ agglutinin (WGA)–Sepharose 4B column. High-affinity [^{3}H]fMet-Leu-Phe binding to the WGA-purified receptor was diminished and exhibited reduced guanine nucleotide sensitivity. High-affinity [^{3}H]fMet-Leu-Phe binding and guanine nucleotide sensitivity were reconstituted upon the addition of purified brain G-proteins. Similar results were obtained when the receptor was reconstituted with brain G-proteins into phospholipid vesicles by gel filtration chromatography. In addition, we also demonstrated fMet-Leu-Phe-dependent GTP hydrolysis in the reconstituted vesicles. The results of this work indicate that coupling of the fMet-Leu-Phe receptor to G-proteins converts the receptor to a high-affinity binding state and that agonist produces activation of G-proteins. The resolution and functional reconstitution of this receptor should provide an important step toward the elucidation of the molecular mechanism of the fMet-Leu-Phe transduction system in neutrophils.

Formylated oligopeptides stimulate chemotaxis, enzyme secretion, superoxide production, and a variety of other cellular responses in neutrophils by interaction with a specific surface receptor (Painter et al., 1984; Aswanikumar et al., 1977; Schiffmann et al., 1975; Korchak et al., 1984). Occupation of this receptor activates a polyphosphatidylinositol-specific phospholipase C, thereby generating two intracellular second messengers: diacylglycerol, which activates protein kinase C, and inositol trisphosphate, which causes a rise in the intracellular Ca^{2+} concentration (Verghese et al., 1985; Bradford & Rubin, 1985; Brandt et al., 1985; Krause et al., 1985; Ohta et al., 1985; Smith et al., 1986). In analogy to various signal-transducing mechanisms (e.g., the hormone-sensitive adenylyl cyclase and the rhodopsin transducing systems), the formylmethionylleucylphenylalanine (fMet-Leu-Phe)[1] receptor appears to be coupled to phospholipase C via a guanine nucleotide regulatory protein (G-protein) which is ADP-ribosylated by pertussis toxin (Verghese et al., 1985; Bradford & Rubin, 1985; Brandt et al., 1985; Krause et al., 1985; Ohta et al., 1985; Molski et al., 1984; Lad et al., 1985). Both immunological and functional studies indicate that the neutrophil G-protein is distinct from G_i, G_o, G_s, and transducin (Falloon et al., 1986; Gierschik et al., 1986; Verghese et al., 1986; Spangrude et al., 1985; Bokoch & Gilman, 1984).

Recent studies have focused on the mechanism by which agonist occupation of the fMet-Leu-Phe receptor induces activaton of phospholipase C in intact neutrophils as well as in isolated plasma membranes (Verghese et al., 1985; Bradford & Rubin, 1985; Brandt et al., 1985; Krause et al., 1985; Ohta et al., 1985; Smith et al., 1986; Molski et al., 1984; Lad et al., 1985). An approach to elucidate the molecular mechanism of the fMet-Leu-Phe signal transduction pathway is the resolution and reconstitution of individual components into phospholipid vesicles. This approach has proven to be extremely useful in the study of the hormone-sensitive adenylyl cyclase and the light-sensitive cGMP phosphodiesterase systems (Cerione et al., 1985, 1986; Fung, 1983, 1985; Levitzki, 1985; Caron et al., 1985). In this work, we have solubilized and reconstituted into phospholipid vesicles the fMet-Leu-Phe receptor coupled to endogenous G-proteins. Coupling was assessed by measuring both the loss of high-affinity binding in the presence of guanine nucleotides and the fMet-Leu-Phe-dependent GTPase activity. Most importantly, we directly demonstrate that high-affinity binding is dependent upon coupling between receptor and G-protein. Resolution of the receptor from endogenous G-proteins caused a loss of high-affinity binding sites as well as the sensitivity of binding to guanine nucleotides; both were restored by exogenous G-proteins. This work represents an important step in the elucidation of the specific mechanism of receptor–phospholipase C coupling in the fMet-Leu-Phe signalling system and could

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[1] Abbreviations: fMet-Leu-Phe, formylmethionylleucylphenylalanine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; WGA, wheat germ agglutinin; PBS, phosphate-buffered saline; DTT, dithiothreitol; G-protein, guanine nucleotide regulatory protein; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Gpp(NH)p, 5′-guanylyl imidodiphosphate; App(NH)p, 5′-adenylyl imidodiphosphate; IBMX, 3-isobutyl-1-methylxanthine; BSA, bovine serum albumin; NAGA, *N*-acetyl-D-glucosamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEI, poly(ethylenimine); G_i, inhibitory guanine nucleotide binding protein; G_o, guanine nucleotide binding protein from brain; G_s, stimulatory guanine nucleotide binding protein; DOPE, dioleoylphosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; PIP_2, phosphatidylinositol bisphosphate.

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serve as a general model for investigating the molecular mechanism of Ca^{2+}-mobilizing receptors.

Experimental Procedures

Materials

Creatine kinase and GTPγS were from Boehringer Mannheim; BA 85 filters were from Schleicher & Scheull; [^{3}H]-fMet-Leu-Phe and [^{35}S]GTPγS were obtained from New England Nuclear; [^{32}P]GTP was from ICN; CHAPS and Extracti-Gel were obtained from Pierce; SDS was from Bio-Rad; lipids were from Avanti Polar-Lipids; Sepharose 4B, Sephadex G-50, and Dextran were obtained from Pharmacia; oyster glycogen, fMet-Leu-Phe, Gpp(NH)p, ATP, App(NH)p, ADP, GDP, GTP, IBMX, NAGA, PEI, activated charcoal, HEPES, phosphocreatine, wheat germ agglutinin, Lubrol PX, octyl-Sepharose CL-4B, and cholic acid were purchased from Sigma; Ultrogel AcA 34 and DEAE-Trisacryl were obtained from LKB.

Methods

Preparation of Plasma Membranes. New Zealand white rabbit neutrophils were harvested by injecting 500 mL of 0.1% glycogen in sterile PBS (pH 7.4) into the peritoneal cavity of rabbits (Becker & Showell, 1972). Twelve hours later, the peritoneal cavity was drained with a percutaneous Teflon catheter. Typically, 2×10^9 cells (90% neutrophils) were recovered. The cells were washed twice with PBS and then resuspended at 10^8 cells/mL in PBS supplemented with 0.1 mM EGTA. Plasma membranes were prepared according to a modification of the procedure described by Lad et al. (1984). Briefly, lysis was affected by sonication for 7 s at 4 °C with a micro-tip probe sonicator at 20 W. The homogenate was layered over a 25% sucrose, 0.1 mM DTT, PBS cushion and centrifuged at 500*g* for 15 min. The supernatant was then layered over a 30% sucrose, 0.1 mM DTT, PBS cushion and centrifuged at 90000*g* for 30 min. The supernatant and the membrane band were collected and diluted by adding an equal volume of H_2O, and the membranes were pelleted by centrifugation at 150000*g* for 30 min. The membrane pellet was resuspended in 0.25 M sucrose and 20 mM Tris-HCl (pH 7.5) at 1.5–2 mg/mL protein and stored at −70 °C until ready for use.

Solubilization of Plasma Membranes. Plasma membranes (1.5–2.0 mg/mL protein) were solubilized in the presence of 20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 1 mM EGTA, and 1% CHAPS (unless otherwise indicated). This mixture was immediately centrifuged at 200000*g* for 30 min, and the supernatant was defined as the detergent-soluble extract.

Wheat Germ Agglutinin Affinity Chromatography. WGA was conjugated to cyanogen bromide activated Sepharose 4B as described by the manufacturer (Pharmacia). The WGA–Sepharose 4B column was equilibrated with 20 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM KCl, 0.1% CHAPS, and 0.2 mg/mL DOPE (buffer A). Membranes solubilized in 0.5–1.0% CHAPS were then diluted 4-fold with 20 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 140 mM NaCl, and 5 mM KCl prior to incubation with the WGA–Sepharose 4B. After being gently stirred for 30 min at 4 °C, the WGA–Sepharose 4B and soluble protein mixture was packed into a column and washed with buffer A. The receptor was eluted with buffer A containing 300 mM NAGA; 1.5-mL fractions were collected, and protein binding, [^{35}S]GTPγS binding, and [^{3}H]-fMet-Leu-Phe binding in the presence and absence of Gpp(NH)p were measured.

Reconstitution of the fMet-Leu-Phe Receptor. Plasma membranes (1.5–2.0 mg/mL protein) were treated with 0.5 μM SDS (unless otherwise indicated) and immediately centrifuged at 200000*g* for 30 min. The pellet was resuspended in 0.25 M sucrose and 20 mM Tris-HCl (pH 7.5) to achieve the original protein concentration. Solubilization of SDS-treated membranes with CHAPS was carried out as described for untreated plasma membranes.

The reconstitution of solubilized membranes into phospholipid vesicles was performed by gel filtration chromatography. Solubilized plasma membranes (200–400 μL) were combined with DOPE at a protein:lipid ratio of 1:10 (w/w). The mixture was diluted to 1 mL with a solution of 140 mM NaCl, 5 mM KCl, and 20 mM HEPES (pH 7.4) and applied to a Sephadex G-50 (medium) column (1.2 × 48 cm). The column had been pretreated with 50 mL of BSA (0.5%) and 0.5 mL of DOPE (20 mg/mL).

Purification of G-Proteins from Bovine Brain. Bovine brain G-proteins (G_i/G_o) were purified by a modification of the method described by Sternweis and Robishaw (1984). Briefly, crude membranes from bovine brain were solubilized in 1% sodium cholate and sequentially chromatographed through DEAE-Trisacryl, Ultrogel AcA 34, and octyl-Sepharose CL-4B columns. The octyl-Sepharose CL-4B column was substituted for the heptylamine-Sepharose column used by Sternweis and Robishaw (1984). Fractions enriched in [^{35}S]GTPγS binding were pooled and dialyzed against 50 mM HEPES (pH 8.0), 1 mM EDTA, 1 mM DTT, and 0.1% Lubrol PX and then stored at −70 °C. The specific [^{35}S]GTPγS binding of the purified protein was 22 pmol/mg of protein, which is similar to that reported by Sternweis and Robishaw (1984). Prior to use, the Lubrol PX was removed by adsorption of the detergent to Extracti-Gel preequilibrated with 10 volumes of 2 mg/mL BSA. G-Proteins (0.2 mg/mL) were incubated for 30 min at 4 °C with 1 mL of Extracti-Gel in a solution of 35 mM HEPES (pH 7.4), 70 mM NaCl, 0.5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 2.5 mM KCl, 0.2 mg/mL DOPE, 0.1% CHAPS, and 0.05% Lubrol PX. The G-proteins were separated from the Extracti-Gel by centrifugation at 750*g* for 10 min.

[^{3}H]fMet-Leu-Phe Binding. Plasma membranes (10–20 μg of protein) were incubated in 100 μL of a reaction mixture containing 140 mM NaCl, 5 mM KCl, 20 mM HEPES (pH 7.4), 1 mM $MgCl_2$, and 1–10 nM [^{3}H]fMet-Leu-Phe (35 cpm/fmol). After a 30-min incubation at room temperature, the reaction was diluted with 2 mL of ice-cold 20 mM HEPES (pH 7.4), 140 mM NaCl, and 5 mM KCl (buffer B) and immediately filtered through Whatman GF/C filters (Williams et al., 1977). The filters were washed 4 times with 2 mL of buffer B, then dried, and counted. The same method was used to measure [^{3}H]fMet-Leu-Phe binding to solubilized membranes except that GF/C filters were pretreated with 0.3% PEI as described (Marasco et al., 1985; Burns et al., 1983). GF/F filters were employed to assay the binding in reconstituted vesicles (Florio & Sternweis, 1985). Scatchard analysis was performed by using Ligand, a nonlinear least-squares regression analysis computer program. The program was developed by Munson and Rodbard (1980) and modified for Apple II by Teicher, McLean Hospital and Mailman Research Center, Belmont, MA.

[^{35}S]GTPγS Binding. The binding of [^{35}S]GTPγS to plasma membranes, solubilized membranes, and reconstituted vesicles was carried out as described (Sternweis & Robishaw, 1984). Briefly, 5–10 μg of protein was incubated in 150 μL of a reaction mixture containing 140 mM NaCl, 5 mM KCl, 20 mM HEPES (pH 7.4), 20 mM $MgCl_2$, 0.1% Lubrol, 1 mM DTT, and 1 μM [^{35}S]GTPγS (1800 cpm/pmol) for 30–60 min

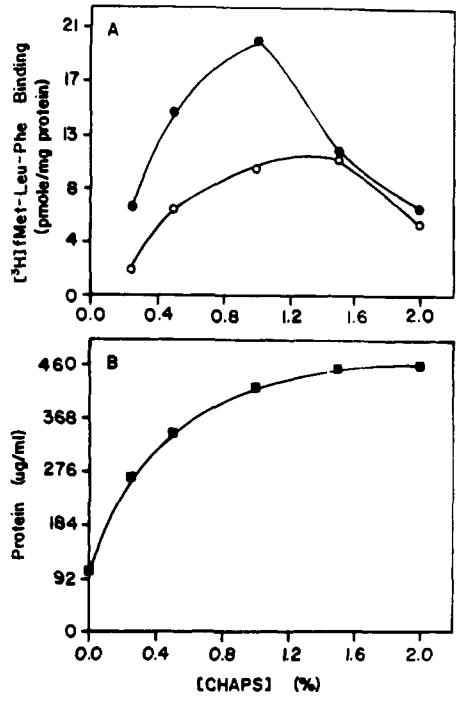

FIGURE 1: Solubilization of the fMet-Leu-Phe receptor. Plasma membranes were solubilized as described under Experimental Procedures with the indicated final concentrations of CHAPS. (A) The soluble fraction was diluted to a final CHAPS concentration of 0.25% with 20 mM HEPES (pH 7.4), 140 mM NaCl, and 5 mM KCl and then assayed for [^{3}H]fMet-Leu-Phe binding in the absence (●) and presence (○) of Gpp(NH)p (10^{-4} M). The final concentration of [^{3}H]fMet-Leu-Phe was 1 nM. (B) The protein concentration of the soluble fraction was measured by using the Bradford assay. The results shown are representative of two separate determinations.

at 30 °C. The reaction was stopped by dilution with 2 mL of 20 mM Tris-HCl (pH 8), 100 mM NaCl, and 25 mM $MgCl_2$ (buffer C) and immediately filtered over BA85 filters. The filters were washed 4 times with 2 mL of buffer C, then dried, and counted.

GTPase Activity. GTPase activity was assayed according to the method described by Okajima et al. (1985). Briefly, 15–20 μg of protein was incubated for 10 min at 37 °C in 25 mM Tris-HCl (pH 7.5), 1 mM EGTA, 0.5 mM App(NH)p, 0.5 mM $MgCl_2$, 1 mM DTT, 0.2 mM IBMX, 0.1% BSA, 5 mM phosphocreatine, 50 units of creatine kinase, and 0–3 μM [^{32}P]GTP (1400 cpm/pmol) in the presence or absence of 1 μM fMet-Leu-Phe as indicated. The reaction was quenched with 1 mL of 20 mM phosphate buffer (pH 7.4) containing 5% charcoal, 0.1% dextran, and 0.5% BSA and then centrifuged at 2500*g* for 10 min; 500 μL of the supernatant was removed and counted.

Protein and Phospholipid Determination. Protein was measured by the Bradford assay (Bradford (1976)), and total phospholipid was determined by the Ames assay (Ames (1966)).

RESULTS

Solubilization and Characterization of the fMet-Leu-Phe Receptor Coupled to Endogenous G-Proteins. To evaluate the optimal conditions for the solubilization of the fMet-Leu-Phe receptor, plasma membranes were treated with increasing concentrations of CHAPS followed by high-speed centrifugation to separate the soluble from the insoluble material. As shown in Figure 1A, at CHAPS concentrations up to 1% the magnitude of [^{3}H]fMet-Leu-Phe specific binding in the soluble extract was proportional to the amount of CHAPS employed. Concentrations of CHAPS above 1% reduced [^{3}H]fMet-Leu-Phe binding without further solubilization of protein (Figure 1B). The detergent concentration for optimal solubilization of the fMet-Leu-Phe receptor was 1%. This concentration of detergent solubilized 50–60% of the receptors and produced 2–3-fold enrichment of specific binding in the solubilized membranes (data not shown).

Table I: Effect of Nucleotides on [^{3}H]fMet-Leu-Phe Binding to Native and Solubilized Membranes[a]

	[^{3}H]fMet-Leu-Phe binding (pmol/mg of protein)	
nucleotide (100 μM)	native membrane	solubilized membrane
none	3.92	5.59
Gpp(NH)p	0.92	1.10
GTPγs	0.71	1.16
GTP	1.00	1.23
GDP	0.87	1.25
App(NH)p	3.72	5.01
ATP	3.34	2.67
ADP	3.50	3.60

[a] [^{3}H]fMet-Leu-Phe binding was assayed in plasma membranes solubilized with 1% CHAPs in the presence of the indicated nucleotide as described under Experimental Procedures. The results are the average of duplicate determinations.

Coupling of the solubilized receptor to endogenous G-proteins was evaluated by measuring the sensitivity of [^{3}H]-fMet-Leu-Phe binding to the nonhydrolyzable GTP analogue Gpp(NH)p. As shown in Figure 1A, Gpp(NH)p inhibited the binding of [^{3}H]fMet-Leu-Phe to the soluble extract when plasma membranes were solubilized with CHAPS concentrations up to 1%. Higher detergent concentrations reduced [^{3}H]fMet-Leu-Phe binding and abolished the inhibition induced by Gpp(NH)p (Figure 1A). These results suggest that the fMet-Leu-Phe receptor remains coupled to G-proteins in detergent solutions when membranes are solublized with CHAPS concentrations lower than 1%.

To determine the specificity of guanine nucleotides in reducing ligand binding, we tested the effects of GTP, GTP analogues, ATP, and ATP analogues on [^{3}H]fMet-Leu-Phe binding (Table I). Ligand binding to soluble and membrane-bound receptor was strongly inhibited by GTP and GTP analogues, and to a lesser extent by ATP analogues or ADP. The concentrations of Gpp(NH)p needed to produce 50% inhibition of fMet-Leu-Phe binding in native membranes and in the soluble extract were 48 and 30 nM, respectively (Figure 2). The effect of Gpp(NH)p on ligand binding was further analyzed by Scatchard plots. In agreement with previous studies (Marasco et al., 1985; Koo et al., 1983), in the absence of guanine nucleotides the Scatchard plot revealed a nonlinear pattern in both solubilized and native membrane preparations (Figure 3). Assuming the existence of two binding sites, the solubilized receptor exhibited a high-affinity site with a K_{d_1} of 0.6 nM and a low-affinity site with a K_{d_2} of 57 nM. These values were not significantly different from those of native membranes (Table II). Scatchard plots of ligand binding in the presence of Gpp(NH)p show a dramatic reduction in the number of high-affinity sites without affecting the dissociation constant (Figure 3 and Table II). In the absence of Gpp-(NH)p, the solubilized membranes exhibited a B_{max} for the high-affinity sites of 4.13 ± 0.44 pmol/mg of protein (mean ± standard error) whereas in the presence of Gpp(NH)p the B_{max} was reduced to 0.73 ± 0.21 pmol/mg of protein. To further characterize the effect of guanine nucleotides on agonist binding, we measured the dissociation rates of bound [^{3}H]fMet-Leu-Phe in the presence and absence of Gpp(NH)p. In Figure 4, we show that Gpp(NH)p accelerates the dissociation of the solubilized [^{3}H]fMet-Leu-Phe reactor complex.

Table II: Scatchard Analysis of [^{3}H]fMet-Leu-Phe Binding in the Presence and Absence of Gpp(NH)p[a]

	native membranes		solubilized membranes	
Gpp(NH)p	-	+	-	+
K_{d_1} (nM)	0.18 ± 0.067	0.25 ± 0.092	0.62 ± 0.092	0.67 ± 0.24
B_{max_1} (pmol/mg)	4.06 ± 0.64	0.55 ± 0.12[b]	4.13 ± 0.44	0.73 ± 0.21[c]
K_{d_2} (nM)	33.4 ± 12.5	53.9 ± 14.4	57.1 ± 14.8	70.15 ± 18.34
B_{max_2} (pmol/mg)	23.8 ± 3.77	17.0 ± 2.75	24.9 ± 2.86	25.4 ± 3.97

[a] Native and soluble membranes were equilibrated with [^{3}H]fMet-Leu-Phe at concentrations between 0.1 and 100 nM in the presence and absence of Gpp(NH)p (10^{-4} M). [^{3}H]fMet-Leu-Phe binding was assayed and analyzed as described under Experimental Procedures. The data are presented as the mean ± standard error. [b] There is a significant difference between native membranes −Gpp(NH)p and native membranes +Gpp(NH)p (Student's t test, $p < 0.05$). [c] There is a significant difference between soluble membranes −Gpp(NH)p and soluble membranes +Gpp(NH)p (Student's t test, $p < 0.05$).

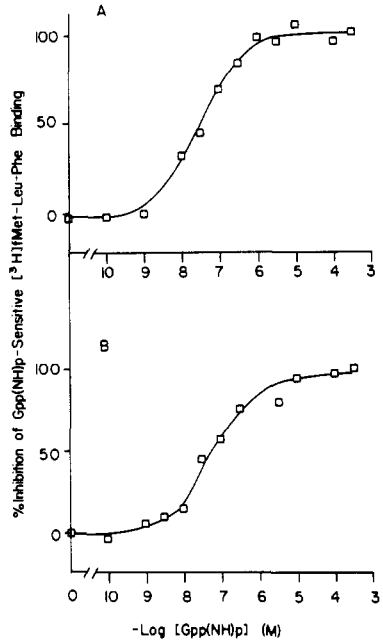

FIGURE 2: Inhibition of Gpp(NH)p-sensitive[^{3}H]fMet-Leu-Phe binding. [^{3}H]fMet-Leu-Phe binding to solubilized (A) and native (B) membranes in the presence of 1 nM fMet-Leu-Phe and the indicated concentration of Gpp(NH)p was performed as described under Experimental Procedures. The results presented are the average of duplicate determinations. The specific binding in native and solubilized membranes was 4.2 and 6.0 pmol/mg of protein, respectively. 100% inhibition is the loss of [^{3}H]fMet-Leu-Phe high-affinity binding obtained at 3×10^{-4} M Gpp(NH)p. This concentration of nucleotide inhibits 75% of the specific binding measured at 1 nM fMet-Leu-Phe.

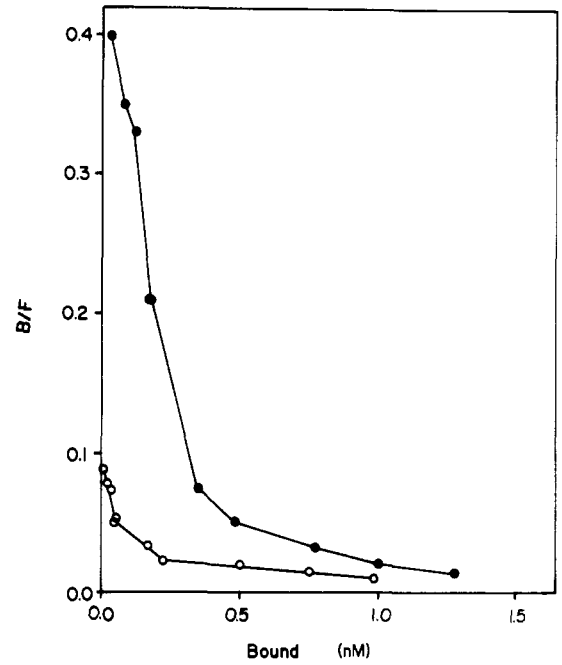

FIGURE 3: Scatchard plot of [^{3}H]fMet-Leu-Phe binding to solubilized plasma membranes in the presence and absence of Gpp(NH)p. Solubilized membranes were equilibrated with [^{3}H]fMet-Leu-Phe at concentrations between 0.1 and 100 nM in the presence (●) and absence (○) of Gpp(NH)p (10^{-4} M). [^{3}H]fMet-Leu-Phe binding was assayed as described under Experimental Procedures. Nonspecific binding was evaluated in the presence of 10 μM unlabeled fMet-Leu-Phe. The data shown are the results combined from two separate experiments.

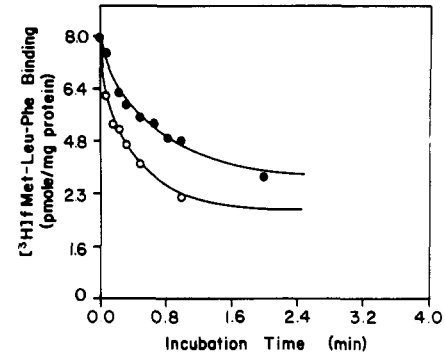

FIGURE 4: Effect of Gpp(NH)p on the dissociation of bound [^{3}H]-fMet-Leu-Phe. Solubilized plasma membranes were equilibrated with [^{3}H]fMet-Leu-Phe for 30 min at 25 °C before the addition of unlabeled fMet-Leu-Phe (1 μM) in the absence (●) or presence (○) of Gpp(NH)p (10^{-4} M). Following the addition of unlabeled ligand, aliquots were removed at the indicated times and assayed for bound [^{3}H]fMet-Leu-Phe as described under Experimental Procedures. The results shown are representative of three separate determinations.

These findings are consistent with the hypothesis that the reduction in high-affinity [^{3}H]fMet-Leu-Phe binding sites by guanine nucleotides is due to the conversion of high-affinity sites to low-affinity sites. Furthermore, our data demonstrate that solubilization of the receptor with CHAPS preserves coupling of the fMet-Leu-Phe receptor to endogenous G-proteins.

Resolution of the fMet-Leu-Phe Receptor from Endogenous G-Proteins and Coupling of the Receptor with Exogenous G-Proteins. To directly determine the role of the G-protein in the modulation of ligand binding to the fMet-Leu-Phe receptor, the receptor was resolved from endogenous G-protein by employing WGA affinity chromatography (Painter et al., 1982). Whereas the receptor was adsorbed to the WGA column, the majority of the G-proteins, identified by [^{35}S]-GTPγS binding activity, were eluted in the flow through. The fMet-Leu-Phe receptor and 10–20% of the total [^{35}S]GTPγS binding activity were eluted with 0.3 M *N*-acetylglucosamine. In order to determine whether the [^{35}S]GTPγS binding proteins eluted with NAGA were functionally coupled to the fMet-Leu-Phe receptor, we evaluated the guanine nucleotide sensitivity of the binding of [^{3}H]fMet-Leu-Phe to the WGA-purified receptor. The ligand binding exhibited little sensitivity to guanine nucleotides. Ribosylation of the WGA-purified

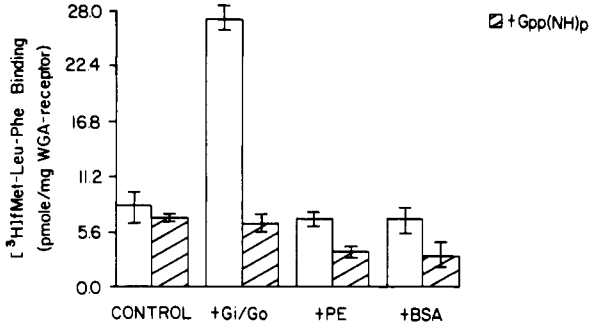

FIGURE 5: Effect of exogenous G-protein on [^{3}H]fMet-Leu-Phe binding. WGA receptor (25 μg) was incubated for 1 h at 4 °C with the indicated solution: (control) 1.8 mL of buffer B; (+G_i/G_o) 50 μg of G_i/G_o, 100 μg of DOPE, and 1.8 mL of buffer B; (+PE) 100 μg of DOPE and 1.8 mL of buffer B; (+BSA) 50 μg of BSA, 100 μg of DOPE, and 1.8 mL of buffer B. Following dilution to a final CHAPS concentration of 0.25%, [^{3}H]fMet-Leu-Phe (10 nM) binding was assayed in the absence (□) and presence of Gpp(NH)p (10^{-4} M). G_i/G_o alone exhibited no specific [^{3}H]fMet-Leu-Phe binding. The results shown are the average of duplicate determinations. Bars define the range.

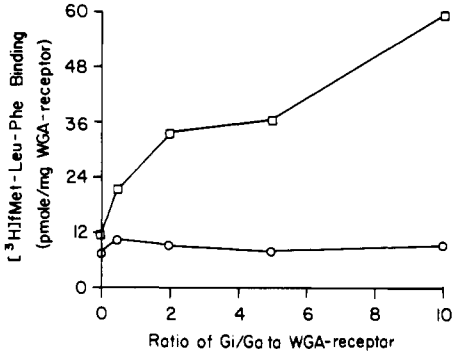

FIGURE 6: Effect of exogenous G_i/G_o concentration on [^{3}H]fMet-Leu-Phe binding. Samples were prepared as described in Figure 5 with solutions containing the indicated ratio of G_iG_o to WGA receptor (w/w). [^{3}H]fMet-Leu-Phe (10 nM) binding was assayed in the absence (□) and presence (○) of Gpp(NH)p (10^{-4} M). The results presented are representative of two separate determinations.

receptor fractions by pertussis toxin showed very little ribosylation of the M_r 40 000 protein compared to unfractionated solubilized membranes (data not shown). These findings indicate that the WGA-purified receptor is uncoupled from endogenous G-proteins.

To evaluate the role of G-proteins in the modulation of the binding properties of the receptor, we added purified bovine brain G_i/G_o to the WGA-purified receptor. As shown in Figure 5, addition of G_i/G_o increased specific [^{3}H]fMet-Leu-Phe binding without increasing nonspecific binding. The G_i/G_o-dependent increase in [^{3}H]fMet-Leu-Phe binding was abolished in the presence of Gpp(NH)p (Figure 5). This effect was specific for G_i/G_o. addition of an equivalent amount of BSA or phospholipid in place of G_i/G_o did not increase [^{3}H]fMet-Leu-Phe equilibrium binding (Figure 5). The magnitude of the increase in [^{3}HfMet-Leu-Phe binding was proportional to the amount of G_i/G_o added. This rise in [^{3}H]fMet-Leu-Phe binding was completely inhibited by Gpp(NH)p at all concentrations of G_i/G_o tested (Figure 6). These findings demonstrate that the high-affinity sites exhibited by the fMet-Leu-Phe receptor are due to coupling of the receptor to endogenous G-protein and that exogenous purified G-proteins from brain can reconstitute the high-affinity binding of the WGA-purified receptor.

Reconstitution of the fMet-Leu-Phe Receptor into Phospholipid Vesicles. Our observation on functional coupling

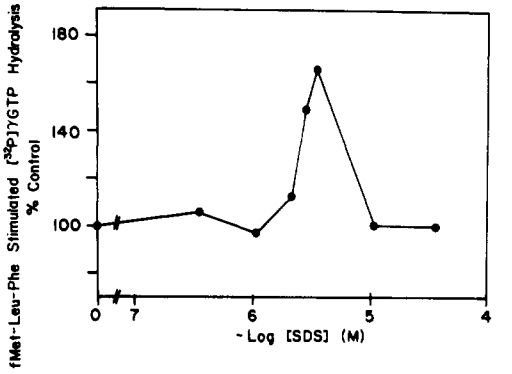

FIGURE 7: fMet-Leu-Phe-stimulated GTPase activity of SDS-treated plasma membranes. [^{32}P]GTP hydrolysis was measured in plasma membranes pretreated with the indicated concentration of SDS as described under Experimental Procedures. The control [^{32}P]GTP hydrolysis was measured in the absence of fMet-Leu-Phe. The results shown are representative of three separate determinations.

between solubilized G-proteins and receptors was extended to a membrane system by reconstituting solubilized membranes into synthetic phospholipid by gel filtration chromatography. Phospholipid vesicles coeluted with [^{3}H]fMet-Leu-Phe and [^{35}S]GTPγS binding activities in the void volume. To demonstrate coupling of the receptor to endogenous G-proteins, Gpp(NH)p-dependent inhibition of [^{3}H]fMet-Leu-Phe binding and fMet-Leu-Phe stimulation of GTPase activity were tested. Although several studies have shown very small increases (10–30%) in fMet-Leu-Phe-dependent GTPase in crude membranes (Okajima et a., 1985; Hyslop et al., 1984; Matsumoto et al., 1986; Feltner et al., 1986), we have found that the fMet-Leu-Phe-dependent GTPase is usually obscured in native plasma membranes because of high basal nonspecific GTPase activity. To address this problem, we have treated the membranes with low concentrations of detergents to reduce the basal nonspecific GTP hydrolysis, thereby revealing fMet-Leu-Phe-dependent GTPase activity. As shown in Figure 7, membranes treated with 2–8 μM SDS exhibited a 60–80% stimulation in GTPase activity in the presence of 1 μM fMet-Leu-Phe. The same concentrations of SDS reduced the basal GTP hydrolysis from 948 to 570 pmol (mg of protein)$^{-1}$ (10 min)$^{-1}$. Lower concentrations of SDS did not affect GTP hydrolysis in the presence or absence of fMet-Leu-Phe, and higher concentrations produced inactivation as well as extensive solubilization of the membranes. Other detergents such as Triton X-100, Lubrol PX, and digitonin failed to affect GTP hydrolysis (data not shown). We therefore used SDS-treated membranes for the reconstitution of the fMet-Leu-Phe receptor.

SDS-treated membranes were solubilized with CHAPS, and the reconstitution of the fMet-Leu-Phe receptor was carried out by gel filtration chromatography. Gpp(NH)p inhibited 70–80% of [^{3}H]fMet-Leu-Phe binding to reconstituted phospholipid vesicles. Further evidence for the coupling of the fMet-Leu-Phe receptor to G-proteins in the reconstituted vesicles is the observation that fMet-Leu-Phe stimulated hydrolysis of [^{32}P]GTP in the reconstituted vesicles to a degree similar to that obtained in SDS-treated membranes. WGA-purified receptor was reconstituted into phospholipid vesicles in the presence and absence of exogenous purified G_i/G_o. Receptors reconstituted in the absence of G_i/G_o exhibited low [^{3}H]fMet-Leu-Phe binding and sensitivity to Gpp(NH)p, whereas in the presence of G_i/G_o the binding was enhanced and Gpp(NH)p sensitivity was restored (Figure 8). These findings indicate that receptor reconstituted into phospholipid vesicles promotes activation of endogenous G-proteins and can

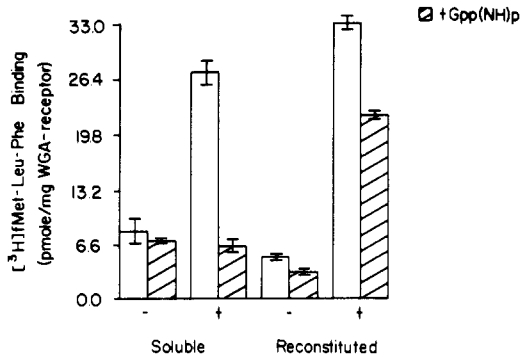

FIGURE 8: [^{3}H]fMet-Leu-Phe binding to soluble and reconstituted preparations of WGA receptor with and without exogenous G_i/G_o. Soluble and reconstituted preparations of WGA receptor (–) or WGA receptor and G_i/G_o (+) were assayed for [^{3}H]fMet-Leu-Phe binding in the presence (hatched bars) and absence (open bars) of Gpp(NH)p (100 μM) as described under Experimental Procedures. The results presented are averages of duplicate determinations. Bars define the range.

be coupled to exogenous G_i/G_o proteins as well.

Discussion

In this study, we have examined the properties of the fMet-Leu-Phe receptor coupled to endogenous G-proteins, and by using resolution and reconstitution methods, we have established that G-proteins are necessary for high-affinity fMet-Leu-Phe binding. This represents the first report of the reconstitution of the high-affinity agonist binding to a resolved fMet-Leu-Phe receptor by purified G-proteins. The hypothesis that a single receptor can exhibit two affinity states and that the transition from high to low affinity is due to uncoupling of the receptor from G-proteins (DeLean et al., 1980) is supported by our data indicating that the WGA-purified receptor exhibited loss of high affinity and lack of guanine nucleotide sensitivity on the binding. This loss of high affinity of the WGA-purified receptor was due to depletion of endogenous G-proteins and not to inactivation of the receptor because complete restoration of the high-affinity binding is achieved by reconstitution with purified G_i/G_o.

It is interesting that G_i/G_o is able to effectively reconstitute high-affinity ligand binding of the resolved fMet-Leu-Phe receptor since this receptor does not appear to be coupled with either of these G-proteins in native membranes. Although there is an M_r 40 000 protein in neutrophil membranes which is a substrate for ADP-ribosylation by pertussis toxin and while pertussis toxin abrogates fMet-Leu-Phe-induced PIP_2 hydrolysis as well as several physiologic functions in neutrophils (Verghese et al., 1985; Bradford & Rubin, 1985; Brandt et al., 1985; Krause et al., 1985; Ohta et al., 1985; Smith et al., 1986; Molski et al., 1984; Lad et al., 1985), the major neutrophil pertussis toxin substrate appears to be distinct from the pertussis toxin substrates G_i and G_o (Falloon et al., 1986; Gierschik et al., 1986; Verghese et al., 1986; Spangrude et al., 1985; Bokoch & Gilman, 1984). Recently, we and other investigators have succeeded in purifying this novel G-protein from neutrophils and have found several biochemical differences as well (Dickey et al., 1987; Oinuma et al., 1987; Neer et al., 1984). The ability of G_i/G_o to reconstitute high-affinity binding is consistent with the finding of Kikuchi et al. (1986) that G_i/G_o was capable of reconstituting fMet-Leu-Phe-induced PIP_2 hydrolysis in pertussis toxin treated neutrophil membranes. It is possible that G_i/G_o are able to functionally couple with the fMet-Leu-Phe receptor similarly to what has been observed in reconstitution experiments involving rhodopsin and G_i (Cerione et al., 1985). The homology among different G-proteins probably underlies this phenomenon. Another possibility is that a minor contaminating G-protein in the G_i/G_o preparation is actually coupling with the fMet-Leu-Phe receptor. Indeed, further purification of G_i/G_o from bovine brain has revealed a minor pertussis toxin substrate of M_r 40 000 distinct from G_i (M_r 41 000) and G_o (M_r 39 000) (Neer et al., 1984). The large molar excess of G_i/G_o over receptor needed in both Kikuchi's et al., 1986) and our reconstitution suggests either that coupling of G_i/G_o with the fMet-Leu-Phe receptor is relatively inefficient or that a large amount of the brain G-protein preparation is needed to achieve adequate levels of the minor M_r 40 000 G-protein responsible for coupling. Indeed, Jones and Reed (1987) have recently identified three G_i isoforms on the basis of cDNA cloning. Whether one of three is specifically coupled to calcium-mobilizing receptors remains to be determined.

The resolution of the fMet-Leu-Phe receptor from endogenous G-protein and the reconstitution of fMet-Leu-Phe high binding affinity with exogenous G-proteins in detergent-solubilized membranes and phospholipid vesicles provide an extremely versatile system for the study of the fMet-Leu-Phe receptor signal transduction mechanism. The elucidation of this mechanism is important not only for the understanding of neutrophil activation but also for the characterization of a receptor–G-protein complex known to be coupled to phospholipase C.

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