Fluorescence Analysis of the Size of a Binding Pocket of a Peptide Receptor at Natural Abundance†

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ABSTRACT: We have studied the topography of interaction of a family of fluorescent formyl peptides containing four (CHO-Met-Leu-Phe-Lys-fluorescein), five (CHO-Met-Leu-Phe-Phe-Lys-fluorescein), and six (CHO-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein and CHO-Met-Leu-Phe-Phe-Phe-Lys-fluorescein) amino acids with their receptor using spectroscopic methods adapted to small sample volumes. Only the fluorescent peptides containing four and five amino acids were quenched upon binding to the receptor, indicating physical contact of the chromophore with the receptor. In contrast, only the hexapeptides were accessible to antibodies to fluorescein. Taken together, these results suggest that the carboxy terminus of the tetrapeptide or the pentapeptide is protected in the receptor binding pocket while the fluorescein on the carboxy terminus of either hexapeptide is exposed and recognized by the antibody to fluorescein. These results indicate that the binding pocket accommodates at least five but no more than six amino acids.

Several strategies have been used to elucidate the interaction between ligand- and membrane-bound signal-transducing receptors. The classical method for defining the binding pocket has relied on the structure–activity and structure–affinity relationships of families of structurally related ligand molecules. More recently, knowledge from amino acid sequences of receptor proteins has suggested that membrane receptors that couple to G proteins may form a family of related molecules which span the plasma membrane with seven α-helical transmembrane domains (Strader et al., 1989). The ligand binding pocket appears to be formed by a cleft located within the membrane-spanning regions. In the case of both the retinal chromophore bound to rhodopsin and the catecholamine binding site of the β-adrenergic receptor, the ligand interacts with amino acid side chains about one-third of the way down from the outer surface of the transmembrane domains (Strader et al., 1989; Findlay & Pappin, 1986).

Membrane signaling receptors that bind peptides are ubiquitous in nature, but there is little information about the nature of the binding pocket. However, ligand molecules covalently modified by fluorescent probes have already proven to be of great utility in studies of the interaction of formyl peptides with their receptor on neutrophils (Sklar et al., 1984, 1989). This receptor is linked through a G protein to cell responses essential in host defense. Structure–function studies indicate that at least four or five amino acids from the amino terminus of the ligand contribute to its activity, suggesting a binding pocket that accommodates molecules of at least that dimension (Niedel et al., 1979; Freer et al., 1982). While previous studies have taken advantage of fluoresceinated ligands as a tool for detecting and quantitating the receptors at natural abundance (Sklar et al., 1984, 1989), in principle, one can also take advantage of the environmental sensitivity of the probes to examine structural features of the ligand–receptor interaction. In this paper we report the environmental fluorescence changes (quenching) associated with ligand binding to the formyl peptide receptor and the accessibility of the FITC[1] chromophore covalently linked to several peptides that bind to the formyl peptide receptor, at receptor concentrations comparable to their natural abundance (1 nM). The binding pocket appears to accommodate no more than six amino acids. These and related approaches should be able to provide extensive information about the binding pocket of many peptide receptor systems.

Materials and Methods

Fluorescent Formyl Peptides. The hexapeptide fNleLFNleYK-fL (FITC isomer I) was prepared as previously (Sklar et al., 1984) or obtained from Molecular Probes (Eugene, OR). The tetrapeptide fMLFK-FL (FITC isomer I) was obtained from Peninsula Laboratories (Burlingame, CA), and fMLFK-FL (FITC isomer II) was prepared as described previously (Seligmann et al., 1984). fMLFFK and fMLFFFK were prepared by the rapid mixed anhydride (Muthukumaraswamy & Freer, 1987) and Merrifield solid-phase (Stewart & Young, 1987) procedures, respectively. Deprotection was with anhydrous HF containing anisole and dimethyl sulfide. Fluorescein was introduced by the reaction of the formylated peptide (0.1 mmol) with 1 equiv of 2 mL of saturated sodium bicarbonate and 0.5 mL of acetonitrile. Reaction was for 2 h at RT, and the reaction was stopped by the addition of 0.1 mmol of ethanolamine. Purification was by HPLC on a Whatman ODS-3 column with gradients of 0.1% trifluoroacetic acid/acetonitrile. Gradients were 0–30% and 30–60% for the FITC derivatives of fMLFFK and fMLFFFK, respectively. Aliquots of these peptides were stored in DMSO at concentrations ranging from 100 μM to 1 mM and quantitated by absorbance spectroscopy as indicated

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[1] Abbreviations: FITC, fluorescein 5′- or 6′-isothiocyanate (isomer I or II, respectively); fMLFK, CHO-Met-Leu-Phe-Lys; fMLFFK, CHO-Met-Leu-Phe-Phe-Lys; fMLFFFK, CHO-Met-Leu-Phe-Phe-Phe-Lys; fNleLFNleYK, CHO-Nle-Leu-Phe-Nle-Tyr-Lys; GTP(S), guanosine 5′-*O*-(3-thiotriphosphate).

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previously (Sklar et al., 1984).

Neutrophils. Human neutrophils were prepared by the elutriation method of Tolley et al. (1987) and permeabilized essentially as described by Smolen et al. (1987), as modified for elutriated cells (Sklar et al., 1987).

Miscellaneous Reagents. The polyclonal antibody to fluorescein was prepared (Sklar et al., 1984), and its high-affinity binding was quantitated as previously described (Levison et al., 1975). The formyl peptide antagonist tBoc-Phe-Leu-Phe-Leu-Phe was obtained from Vega Biochemicals (Tucson, AZ). GTP(S) was obtained from Sigma (St. Louis, MO).

Spectroscopic Binding Methods. The analysis of the interaction of formyl peptides with their receptors on permeabilized cells followed the methods of Sklar et al. (1984), now adapted to small volumes. In these experiments, round glass cuvettes (i.d. 6 mm, Sienco, Inc., Morrison, CO) contained 200 μL of neutrophils (10^7 cells/mL) in 120 mM KCl, 20 mM NaCl, 10 mM HEPES, 1 mM EGTA, 5 mM MgCl, and 1 mM PMSF, pH 7.4. The cuvettes were mounted in the light path of the SLM 8000 with an adapter similar to the aggregometer adaptor available from Sienco. The cuvette was stirred by a 2 × 5 mm Teflon-coated magnet (Bel Arts, Pequannock, NJ) and thermostated to 37 °C. Sample addition was through a pinhole in the top of the sample compartment with Hamilton (Reno, NV) syringes (5–25 μL). In most cases, 2 μL of reagent was added. Sample mixing (~1 s) in this system was consistently more uniform and rapid than that in square cuvettes. Excitation was at 490 nm with a 490 nm, 10 nm band-pass interference filter (Corion, Holliston, MA). Emission was detected through a Corning 3-70 cut-on filter (VWR Scientific, San Francisco, CA) and a 520 nm, 10 nm band-pass interference filter (Corion, Holliston, MA).

Results and Discussion

Binding of fluoresceinated peptides to the formyl peptide receptor has previously been detected with antibodies to fluorescein which discriminate between receptor-bound and free peptide. The antibody to fluorescein has been shown to rapidly quench the free ligand with about 90% efficiency at a near-diffusion-limited rate (10^8 M^{-1} s^{-1}) while the receptor bound ligand was shown to be comparatively poorly recognized (Sklar et al., 1984), if at all.[2] All four of the peptides bind with high affinity (K_d < 1 nM) to the receptor (Figure 1, column A) in the form of a slowly dissociating complex in the absence of guanine nucleotide. This high-affinity binding is identified as the ternary complex of ligand, receptor, and G protein by the sensitivity of ligand binding to guanine nucleotide, which is known to cause dissociation of the ternary complex and formation of the rapidly dissociating binary ligand–receptor complex (Sklar et al., 1987).

As the tetrapeptide and pentapeptide bind, their fluorescence is quenched.[3] Reversible binding and quenching is verified in the absence of antibody in Figure 1 (column B) by showing that the addition of a competitive inhibitor in the presence of guanine nucleotide rapidly restores the fluorescence intensity to the control (unbound) level. Since the binding of the fluorescent ligand is high affinity in the absence of guanine nucleotide, it is not rapidly displaced by unlabeled ligand until the guanine nucleotide is added. Identical results are detected with cell membranes, and a similar pattern is followed in intact cells; however, GTP(S) in intact cells does not release the bound ligand because the intracellular compartment is not accessible to GTP(S). These results suggest that the environment of the fluorescein of the tetrapeptide and the pentapeptide is altered by binding to the receptor while that of the hexapeptide is not, possibly because the fluorescein in the shorter peptides "sees" and interacts with the receptors, while that on the longer peptides does not.

In Figure 1, column C, we used antibody to fluorescein to probe the accessibility of the bound ligand by varying the antibody concentration. With the tetrapeptide and the pentapeptide, recognition of the receptor-bound ligand was not a function of the amount of the antibody added. With antibody in excess, the rate of recognition of the chromophore by the antibody is limited to the natural dissociation rates (half-time ~300 s) of these ligands from the ternary complex (see, for example, dissociability after the addition of nonfluorescent competitor alone in Figure 1, column B). In contrast, for the receptor-bound hexapeptides, the rate of recognition and quenching of the fluorescein by the antibody depends upon the amount of antibody. The proportions of the free (rapidly quenched) and receptor-bound (slowly quenched) peptide were not dependent on the amount of antibody present. Analysis of initial rate data (Figure 2) yields a rate constant of 1×10^5 M^{-1} s^{-1} for antibody recognition of the bound ligand. The magnitude of the rate is typical of antibody binding to membrane binding sites (Erickson et al., 1987). The data demonstrate that antibody recognizes the fluorescein of both hexapeptides when they are bound to the receptor but fails to recognize either of the shorter peptides.

Estimates of the size of the receptor binding pocket may be inferred from the extent of the ligand accessible to the antibody binding pocket. Using divalent haptens and polyclonal dinitrophenyl antibodies, Wilder et al. (1975) estimated the depth of an antibody combining site to be 10–13 Å. Recent crystal structure data (Herron et al., 1989) suggest that fluorescein occupies a slot in its antibody about 9 Å deep, with only the benzoyl ring (which is linked to the peptide) exposed to solvent at an open end of the slot. Taken together, these data obtained for fluorescence quenching, ligand accessibility, and antibody structure provide a basis for the simplistic diagram of the receptor binding pocket detailed in Figure 3, upper panel. The carboxy termini including the fluorescein of the two shorter peptides are shown within the binding pocket of the receptor, while the fluoresceins of the longer peptides are not in contact with the receptor and are outside of the pocket accessible to the antibody. Because the formyl group is believed to contact a hydrophobic region in the pocket (Freer et al., 1982), it is shown as buried.

There are several common mechanisms of fluorescein including protonation (Mercola et al., 1972) or contact with protein tryptophan residues (Watt & Voss, 1977). We have observed a quenching efficiency[4] of 52 ± 16% (10 donors) for the pentapeptide and 34 ± 12% (12 donors) for the tetra-

[2] In previous studies using 25 nM antibody, we indicated that recognition of the hexapeptide bound to receptor was limited by its rate of dissociation from the receptor. In fact, the rate of recognition of this ligand by 25 nM antibody is almost identical with the rate of dissociation of this ligand from the receptor. By working with small sample volumes, it has been practical to increase the antibody concentration and to increase the rate of recognition.

[3] Analysis of the concentration dependence of binding yields an association rate constant of ~2×10^7 M^{-1} s^{-1}, which approaches the diffusion limit for small ligand interactions with membrane receptors (Fay and Sklar, unpublished results).

[4] Quenching is calculated (Figure 1, column A, top panel) by evaluating the fluorescence of free ligand (point a), free and receptor-bound ligand (point b), receptor-bound and antibody-bound ligand (point c), and antibody-bound ligand (point d). The quenching is calculated as $1 - (c - bd/a)/(a + c - b - d)$ (Fay and Sklar, unpublished data).

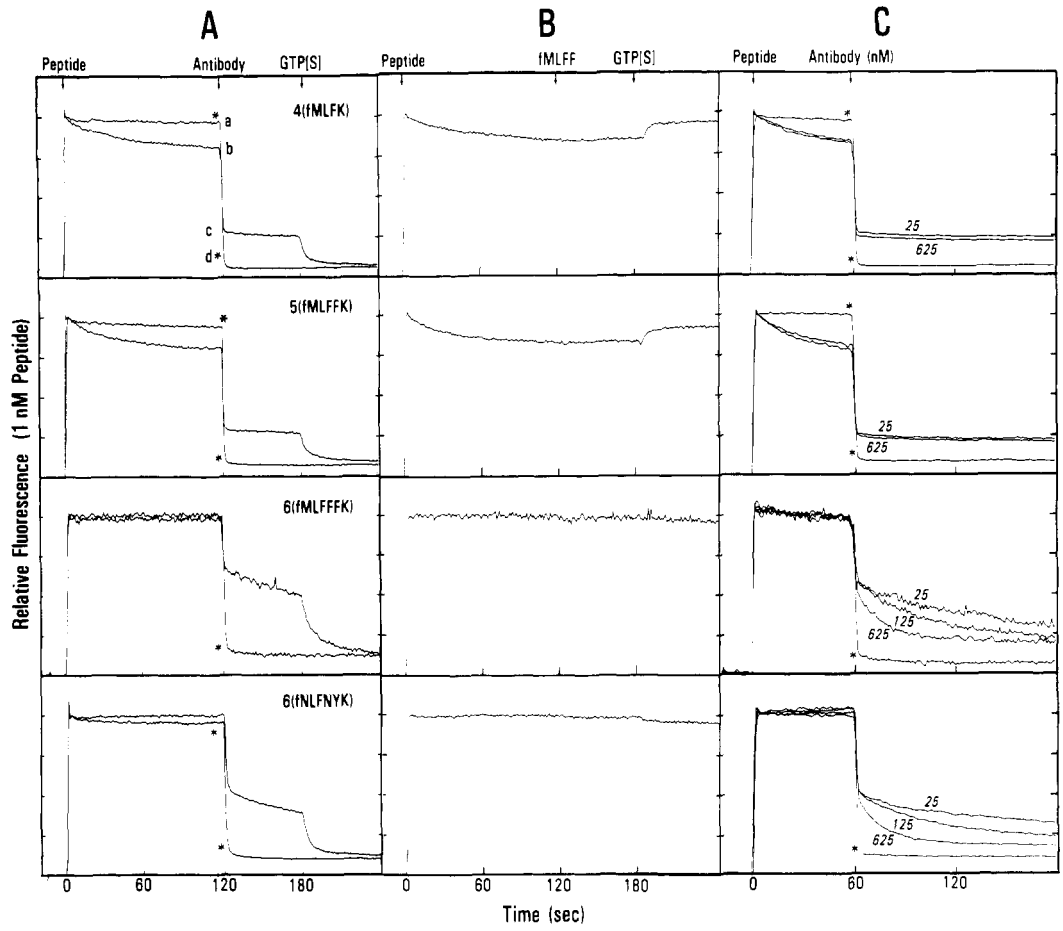

FIGURE 1: (Column A) Discrimination of receptor-bound peptide on permeabilized neutrophils by antibody to fluorescein. Data are displayed as the fluorescence intensity of 1 nM fluorescent peptide vs time. The panels show fMLFK-FL, fMLFFK-FL, fMLFFFK-FL, and fNleLFNleYK-FL. The background fluorescence intensity and light scatter signal of the cells which is typically 10% of that of the signal and constant throughout the experiment has been subtracted from the data. The full-scale fluorescence value of the peptide is 10^5 photons/s, and for comparison, the fluorescence of the different peptides has been normalized to the same scale. At time 0, 1 nM fluorescent peptide was added; at t = 120 s, 25 nM antibody was added; at t = 180 s, GTP(S) (10^{-4} M) was added. The control samples (*) were preincubated with excess receptor antagonist (10^{-5} M tBoc-Phe-Leu-Phe-Leu-Phe). Points a and d represent the fluorescence intensity of the control sample (no receptor binding) before and after antibody addition while points b and c represent the fluorescence intensity of the sample in which receptor binding occurred before and after antibody addition. The asterisks are shown at points a and d for the tetrapeptide and pentapeptide and at point d for the hexapeptides, except in the lower panel where it is shown also at point a for the control sample. Samples contained 2 million permeabilized cells in 200 μL, yielding a receptor concentration of $\sim$1 nM. Additions are indicated by the arrows. (Column B) Binding and quenching of peptides on permeabilized cells. At time 0, the peptide is added. At t = 120 s, the nonfluorescent agonist fMLFF (10^{-6} M) was added. At t = 180 s, GTP(S) (10^{-4} M) was added. (Column C) Effect of antibody concentration of the fluorescence of the peptides in permeabilized cells. Fluorescent peptide was added at time 0; antibody (25, 125, or 625 nM) was added at t = 60 s. The small intensity differences with 25 or 625 nM antibody for the tetrapeptide and pentapeptide arise due to sample dilution. The sample is diluted 1% at 25 nM and 5% at 625 nM. Control samples are indicated by the asterisks (*) at points a and d for the tetrapeptide and pentapeptide and only at point d for the hexapeptides.

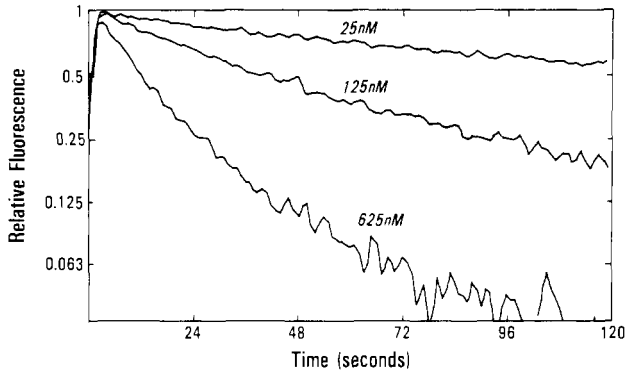

FIGURE 2: Rate of quenching for fNleLFNleYK-FL vs antibody concentration. The data are a plot of the inital rate of antibody binding from Figure 1, column C. The analysis of the data (not shown) shows that the rate of recoginition of the receptor-bound hexapeptide is 10^5 M^{-1} s^{-1}.

peptide. The differences for the two ligands could represent a charge gradient or variable distances from a tryptophan in

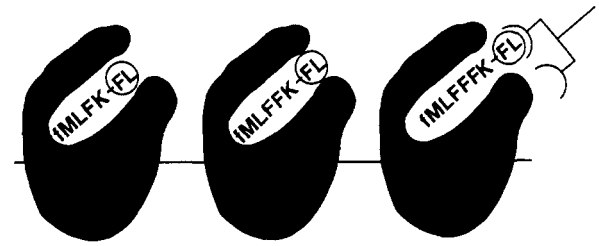

FIGURE 3: Schematic diagram of the binding pocket of the receptor. Shorter peptides are shown with fluorescein in physical contact with the pocket; longer peptides are shown with fluorescein out of the pocket accessible to antibody to fluorescein.

the binding pocket. It is worth noting that regions of positive charge in the binding pocket of the formyl peptide receptor have been previously suggested (Freer et al., 1982) including a histidine, a possible proton donor at neutral pH which influences ligand binding (Spilberg et al., 1986). A standard curve of quenching of the pentapeptide as a function of pH indicates that 52% quenching would correspond to an apparent

pH change from 7.0 for the unbound peptide to 6.2 for the bound species, but it has not yet been possible to define the mechanism of quenching at natural abundance by spectroscopic methods.

Our results represent the first analysis of the size of the binding pocket of a membrane receptor by fluorescence techniques. The experiments used fluorescein as a probe because it can be detected at natural abundance of the receptors and antibodies to fluorescein are well characterized. The spectral sensitivity of other probes will permit additional characterization of the pocket. The challenge for future investigations will be to improve the simplistic view of Figure 3 by extending the molecular resolution of the analysis. This includes determining the depth, width, orientation, distance from the membrane, flexibility, and charge structure within the binding pocket. These details must then be related to the sequence and tertiary structure of the receptor, particularly with respect to the putative cleft formed by the transmembrane domains. These initial observations indicate that it will be possible to map the topography of interaction between ligands and peptide receptors by use of fluorescent probes attached to peptides of systematically varied structure. The methods will also require the application of quenching agents of different size to probe accessibility and of spectroscopic analysis to characterize the local environment of the probes. These methods are suitable for receptors of lower density (tens of thousands per cell) or lower affinity (K_d > 1 nM) than the formyl peptide receptor by use of samples in which the total receptor concentrations are high enough to permit binding of a large fraction of the fluorescent ligand. With the formyl peptide receptor and high-affinity ligands, it is possible to examine the binding pocket at conditions similar to natural abundance (i.e., 1 nM). In typical sample volumes of 200 μL, observations are made on 200 fmol of receptor.

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