Extensive Contact between G_{i2} and N-Formyl Peptide Receptor of Human Neutrophils: Mapping of Binding Sites Using Receptor-Mimetic Peptides†

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ABSTRACT: The N-formyl peptide receptor (FPR) of human neutrophils is a member of the G protein-coupled receptor (GPCR) superfamily. Sites on agonist-occupied FPR involved in binding the G_{i2} protein were investigated by competition with synthetic receptor-mimetic peptides. Twenty-three synthetic FPR-mimetic and control peptides were tested for their ability to disrupt functionally active complexes of FPR and G_{i2} in octyl glucoside, assayed by changes in sedimentation rates of FPR in detergent-containing sucrose gradients. GPCRs are thought to contain seven transmembrane segments with three cytoplasmic connecting loops and a cytoplasmic tail. Only certain peptides from regions in or adjoining each of the four predicted cytoplasmic domains of the 350 amino acid FPR, *including the first cytoplasmic loop,* were able to disrupt the reconstituted FPR−G_{i2} complex. The IC_{50}s of the peptides that were able to fully disrupt the FPR−G_{i2} complex ranged from 20 μM (C2W 122−134) to 1.4 mM (C3A 230−246), a range similar to peptide inhibition of other G protein-coupled receptor−G protein interactions. Detergent concentrations above and below the critical micelle concentration had no effect on the activity of even the most hydrophobic peptide, C3B, and there was no apparent correlation of activity with hydrophobic moment, hydrophilic index, or net charge of the peptides. Control peptides from irrelevant proteins with similar physical properties and FPR extracellular domains did not dissociate the reconstituted FPR−G_{i2} complex up to 5 mM, the highest concentration tested. These results suggest that the structural interface between FPR and G_{i2} is extensive, requiring at least six interfacial, intramembrane, and cytoplasmic domains of FPR but without favorable interaction of the hydrophilic cytoplasmic loops connecting predicted transmembrane helices 3−7.

Neutrophils act as the first line of defense against the invasion of microorganisms in the body. Bacterial metabolites such as N-formylmethionyl peptides are chemoattractants for neutrophils and bind to specific surface receptors that trigger specific host defensive processes (Allen et al., 1988; Boulay et al., 1990a; Williams et al., 1977). A primary molecular event, mediated by the agonist-occupied N-formyl peptide chemoattractant receptor (FPR), is the induction of GDP−GTP exchange on heterotrimeric G proteins (Snyderman & Uhing, 1988; Snyderman et al., 1986; Bokoch, 1990; Bokoch & Gilman, 1984). FPR cDNA has been cloned and sequenced (Boulay et al., 1990a), and hydropathy analysis of the derived amino acid sequence and conserved amino acids (Hargrave & McDowell, 1992; Probst et al., 1992; Baldwin, 1993) identify FPR as a member of the G protein-coupled receptor (GPCR) superfamily (Dohlman et al., 1991). A striking feature of GPCR is the presence of seven predominantly hydrophobic regions, which are thought to form membrane-spanning α-helices, connected by relatively hydrophilic polypeptide domains on the extracellular and the cytoplasmic sides of the receptors. The seven transmembrane helix motif was found by electron diffraction analysis of bacteriorhodopsin, a non-G protein-linked light-driven proton pump (Henderson & Unwin, 1975). Many of the salient features of the bacteriorhodopsin transmembrane motif are thought to be present in rhodopsin and other GPCR (Dratz & Hargrave, 1983), and this has been supported in the case of rhodopsin (Schertler et al., 1993) and β2-adrenergic receptor [reviewed in Dohlman et al. (1991)]. Based on sequence homology between FPR and other GPCR, working models of the transmembrane organization of FPR have been proposed (Boulay et al., 1990a; Bommakanti et al., 1993; Ye et al., 1993).

The photoaffinity agonist receptor migrates as a broad band between 50 and 70 kDa (Niedel et al., 1980; Schmitt et al., 1983; Allen et al., 1986a) on denaturing sodium dodecyl sulfate−polyacrylamide gels. Nonglycosylated FPR, expressed in *Escherichia coli* (Lala et al., 1993), and the enzymatically deglycosylated native FPR (Malech et al., 1985) migrate as sharp 35-kDa bands on SDS−polyacrylamide gels.

When neutrophil plasma membranes are solubilized in octyl glucoside (or Triton X-100), photoaffinity agonist-labeled FPR exhibits two size forms with apparent sedimentation coefficients of approximately 4 and 7 S. The two forms can be resolved by velocity sedimentation in linear sucrose density gradients (Jesaitis et al., 1989). The more rapidly sedimenting 7S form can be converted to the 4S form by inclusion of GTPγS in the solubilization buffer. Detergent-solubilized FPR retains the capacity for interaction with added G protein(s), in a concentration-dependent manner,

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Table 1: Summary of the Peptides Used in the Present Study of Peptide-Induced Disruption of FPR−G_{i2} Complexes[a]

peptide name	sequence and position in FPR	IC_{50}	length	net charge	hydrophilic index	hydrophobic moment μ
C1W	43-GNGLVIWVAGFRMTHTVTT-61	100 μM	19	+1	−0.86	0.20
C2A	134-QNHRTVSLAKKVIIGPW-150	100 μM	17	+3	−0.23	0.20
C2M	127-VLHPVWTQNHRTVS-140	inactive	14	+1	−0.65	0.25
C2B	119-IALDRCVCVLHPVWT-133	1 mM	15	0	−0.82	0.21
C2W	122-DRCVCVLHPVWTQNHRTVSLAKK-144	20 μM	23	+3	−0.08	0.26
C2W++	119-IALDRCVCVLHPVWTQNHRTVSLAKKVI-146	0.5 mM	28	+3	−0.35	0.25
rC2W	144-KKALSVTRHNQTWVPHLVCVCRD-122	2 mM	23	+3	−0.08	0.27
C3A	230-KQGLIKSSRPLRVLSFV-246	1.4 mM	17	+4	+0.03	0.36
C3M	227-KIHKQGLIKSSRP-239	inactive	13	+4	+0.63	0.35
C3B	210-FSAPMSIVAVSYGLI-224	0.5 mM	15	0	−1.12	0.15
rC3A	246-VFSLVRLPRSSKILGQK-230	>10 mM	17	+4	+0.03	0.31
CTA	301-YVFMGQDFRERLIH-314	insoluble	14	0	−0.17	0.26
CTB	304-MGQDFRERLIHALPAS-319	3 mM	16	+1	+0.13	0.20
CTC	315-ALPASLERALTEDST-329	3 mM	15	0	+0.38	0.22
CTD	330-QTSDTATNSTLPSAE-344	inactive	15	−2	+0.22	0.15
CTE	322-RALTEDSTQTSDTAT-336	400 μM	15	−1	+0.62	0.20
CTZ	337-NSTLPSAEVALQAK-350	inactive	14	0	0.00	0.26
rCTE	336-TATDSTQTSDETLAR-322	inactive	15	−1	+0.62	0.21
E1A	85-KAMGGHWPFGWFLCK-99	inactive	15	+2	−0.84	0.22
E2A	170-KTGTVACTFNFSPWT-184	inactive	15	+1	−0.73	0.19
gp91 phox	296-KVVITKVVTHPFKTIE-306	inactive	16	+1	−0.13	0.30
gMKI	53-MKIIHEDGYS-62	inactive	10	−1	+0.20	0.27
aMKI	190-MKILTERGYS-199	inactive	10	0	+0.21	0.32

[a] Peptide sequences were derived from the known amino acid sequence of FPR (Boulay et al., 1990b). The alphanumeric names of the FPR peptides used are a shorthand to denote their disposition in the preliminary topological model we proposed for FPR (Bommakanti et al., 1993): C2 for second cytoplasmic loop, E1 for first extracellular loop, CT for carboxyl-terminal tail, etc. The middle regions of the predicted loops and entire predicted loop sequences are denoted by M and W, respectively. Control peptide $gp91^{phox}$ was from neutrophil cytochrome *b* heavy chain, while peptides g-MKI and g-MKI are from human $G_{i2\alpha}$ subunit and human cytosolic β-actin, respectively. The numbers flanking the first and last residues of the peptides correspond to their location in the human FPR amino acid sequence. The peptides were assayed for their ability to dissociate reconstituted FPR-G_{i2} 7S complexes as described in the Materials and Methods section and in the legends for Figures 1 and 2. Peptide concentrations required to dissociate 50% of the 7S FPR-G_{i2} complexes were measured from peptide titration plots such as those shown in Figure 2 and tabulated as IC_{50} values. The net charge (at neutral pH), the calculated hydrophilic index (Hopp & Woods, 1981), and hydrophobic moment (Eisenberg et al., 1984) values for the peptides are tabulated.

upon reconstitution into phospholipid vesicles (Cupo et al., 1989) or in detergent extracts (Bommakanti et al., 1992). The 7S form can be produced from 4S FPR by adding G_{i2}, and the reconstituted 7S is quantitatively dissociated to 4S by guanine nucleotides (Bommakanti et al., 1992) and is immunosedimentable by anti-$G_{i2\alpha}$ antibodies (Bommakanti et al., 1992). The 7S form of FPR is a functional, physical complex with the G_{i2} protein since it contains agonist occupied receptor and an *empty* guanine nucleotide binding site (Bommakanti et al., 1994) required for receptor-mediated GDP release and GTP exchange.

Study of the contact sites responsible for GPCR and G protein coupling is being actively pursued using a variety of approaches including site-directed mutagenesis (Savarese & Fraser, 1992), construction of chimeric receptors (Schwinn et al., 1992), and application of synthetic peptides to block and map putative interactive regions (Hamm, 1991; Hargrave & McDowell, 1992; Hamm et al., 1988; Dratz et al., 1993). Each method has disadvantages, which include perturbation of global or remote structural units (site-directed mutagenesis and chimera construction) and improper folding or possible nonspecific binding of synthetic mimetic peptides. Together, however, they allow a structural consensus to be built that is providing important clues about the structure and function of GPCR and their signal transduction partners.

Studies with rhodopsin (Khorana, 1992; Hargrave & McDowell, 1992; Hamm et al., 1988), muscarinic receptors (Bonner, 1992), and adrenergic receptors (Schwinn et al., 1992) suggest that receptor regions involved in G protein coupling include parts of the second intracellular loop, amino and carboxyl portions of the third loop, and the portion of the C-terminus closest to the membrane. The $\beta\gamma$ subunits as well as the carboxyl- and amino-terminal regions of the α subunits of heterotrimeric G proteins have been shown to mediate receptor interaction (Hamm et al., 1988; Palm et al., 1990) and contribute to receptor−G protein specificity (Kleuss et al., 1992, 1993; Lefkowitz, 1992). However, little is known about participation of the first predicted cytoplasmic loop or membrane domains of GPCR. The structural implications of multisite interaction have also received relatively little attention.

In the current study, we investigated a much wider range of the putative cytoplasmic and intramembrane regions of FPR that might be involved in G_{i2} binding, using receptor-mimetic peptide competition. When our current results, along with our other previous findings on FPR (Bommakanti et al., 1993), are interpreted using an experimentally supported consensus model of GPCR (Baldwin, 1993), they suggest that contact between agonist-occupied FPR and G_{i2} is extensive and may involve participation of hydrophobic sequences predicted to be intramembrane but without favorable interactions with the hydrophilic loops connecting the predicted transmembrane α-helical barrels.

MATERIALS AND METHODS

Buffers, chemicals, and methods of cell preparation were as previously described (Jesaitis et al., 1989; Parkos et al., 1987). Chemicals used for G protein isolation were as described by Bokoch et al. (1988).

Peptide Synthesis and Characterization. The peptides referred to in Table 1 of the Results section were synthesized

and characterized as described previously (Bommakanti et al., 1993). The peptides were purified by high-performance liquid chromatography in gradients of acetonitrile/6 mM HCl in water, and the purity of the fractions was assessed by electrospray mass spectrometry in methanol/water/acetic acid (50/50/1). All peptides utilized were estimated to be at least 95% pure. Peptide stock solutions were 25−100 mM in the 1% octyl glucoside-containing extraction buffer with 0.02% sodium azide, and the pH was adjusted to 7.5. The sequence, certain physicochemical properties of the peptides [hydrophilic index (Hopp & Woods, 1981), hydrophobic moment (Eisenberg et al., 1984), and net charge at neutral pH] are given in Table 1. The positions of the peptides in the sequence of the receptor are depicted in the insets to Figure 2.

Preparation of G Protein. G_i was purified from bovine brain as previously described (Sternweis & Robishaw, 1984) with the following modifications. For the first two chromatographic steps Q-Sepharose Fast Flow ion-exchange medium (Pharmacia) and Sephacryl 200-HR gel-filtration medium (Pharmacia) were used, respectively. G_i was separated from G_o on a 20-mL DEAE-Sephacel column using 0.1% Lubrol in TED buffer as described previously (Bommakanti et al., 1993). Purity was about 95% as assessed by immunoblotting using $G_{i2\alpha}$-specific anti-peptide antibodies and SDS−PAGE followed by silver staining. Nucleotide binding to purified preparations showed 0.90 ± 0.2 mol of GTPγ^{35}S bound/mol of protein, as assessed by the BCA protein assay (Pierce, Rockford, IL). $G_{i2\alpha}$-specific anti-peptide antibodies, as well as $G_{s\alpha}$ and $G_{o\alpha}$ (subtypes 1 and 2) antibodies, were a gift of Dr. David Manning (University of Pennsylvania).

Peptide Effects on Reconstitution of FPR with G_i. Preparation of buffers, neutrophil plasma membranes, photoaffinity agonist labeling of FPR, octyl glucoside extraction, and peptide incubations were carried out essentially as described previously (Allen et al., 1986b, 1989; Bommakanti et al., 1994; Bommakanti et al., 1993). Briefly, octyl glucoside extracts of purified plasma membranes, containing the 4S form of FPR, were divided into aliquots and G_i was added to a final concentration of 250−430 nM. The concentration of FPR in the incubation mixtures was estimated to be about 50−70 nM, assuming 50−70% recovery of FPR from whole cells in the crude extracts. The FPR−G_{i2} mixtures were incubated for 1−2 h on ice and then different concentrations of the peptides were added. The peptide-containing mixtures were further incubated for 1−2 h on ice before layering on preformed linear sucrose density gradients (700 μL, 5−20%) containing 1% octyl glucoside. Gradients were prepared by sequential layering of 5%, 10%, 15%, and 20% sucrose and allowing diffusion for 10 h at 4 °C to achieve linearity. The gradients contained the same concentrations of peptide as were used in the corresponding incubation mixtures to maintain equilibrium dissociation of complexes during sedimentation. Longer incubations with the peptides or preincubation of the peptides with G_{i2}, before addition of FPR, gave identical results, thus indicating that these experiments were effectively conducted at equilibrium.

The gradients were centrifuged in a SW55 Beckman swinging-bucket rotor for 10 h at 45 000 rpm at 4 °C, and receptor peaks were localized after fractionation of the gradients into 55-μL fractions. Individual fractions were subjected to SDS-PAGE, and the receptor content was determined by receptor-specific agonist radioactivity using phosphor technology and phosphor image analysis (Johnston et al., 1990), with a Molecular Dynamics (Sunnyvale, CA) 400E Phosphor Imager and Image Quant software. Sedimentation experiments were calibrated with protein standards by centrifuging a mixture of 10 μg each of cytochrome *c* (2.1 S), bovine serum albumin (4.4 S), porcine immunoglobulin G (7.7 S), and bovine catalase (11.2 S) in parallel with experimental gradients.

RESULTS AND DISCUSSION

Synthetic peptides have previously been shown to interfere with functional (Hamm et al., 1988; Takemoto et al., 1986; König et al., 1989; Münch et al., 1991; Dalman & Neubig, 1991, Schreiber et al., 1994) and physical (Bommakanti et al., 1993; Saxon et al., 1994) receptor−G protein coupling in FPR as well as other GPCR systems. Peptides that are effective in blocking the GPCR−G protein interaction are believed to mimic interfacial contact sites between the proteins. This peptide competition paradigm, in fact, now serves as a standard approach to identifying protein interactive pairs and their recognition sequences (Hamm et al., 1988; Dedman et al., 1993; Smith & Scott, 1993; Houghten et al., 1992).

We have previously shown that a 15 amino acid peptide from the middle of the carboxyl-terminal tail of FPR (CTE) specifically interferes with complex formation between agonist-occupied FPR and G_{i2} (Bommakanti et al., 1993). In contrast, we found that the hydrophilic, putative central regions of the second and third cytoplasmic FPR loops were totally inactive (Bommakanti et al., 1993). In the present work, we investigated 13 additional peptides designed to explore the participation of juxtamembrane regions of the predicted first, second, and third cytoplasmic loops, and the carboxyl-terminal tail. Peptides were synthesized that mimicked overlapping regions that were shown by us and others to be inactive or active or that had not been tested for blocking the FPR-G_i interaction.

Separation of Complexed and Uncomplexed FPR. Covalent labeling of FPR with radiolabeled agonist allows the study of FPR−G protein complexes without purification of FPR (Bommakanti et al., 1992, 1993, 1994; Allen et al., 1986a). Our assay, employing velocity sedimentation in detergent-containing linear sucrose density gradients, measured the amount of FPR complexed with G_{i2} as a function of peptide concentration. Quantitation of the radioactive agonist-labeled FPR in different fractions of the sucrose gradients was achieved using SDS−polyacrylamide gel electrophoresis and storage phosphor image analysis (Johnston et al., 1990). Figure 1 shows the sedimentation profile of agonist-labeled FPR under three different conditions. Panel A shows the sedimentation profile of the G protein-free, 4S form of FPR obtained from GTPγS-treated neutrophil membranes (Bommakanti et al., 1992, 1993). Panel B shows the peak shift in the sedimentation of the agonist-labeled FPR in the presence of added exogenous G_{i2}, which corresponds to the 7S form of FPR that represents a functionally active physical complex of agonist-occupied FPR and G_{i2} (Bommakanti et al., 1992, 1993, 1994; Jesaitis et al., 1989; Schreiber et al., 1993).

The disruptive effect of adding a peptide corresponding to the carboxyl-terminal end of the putative third cytoplasmic

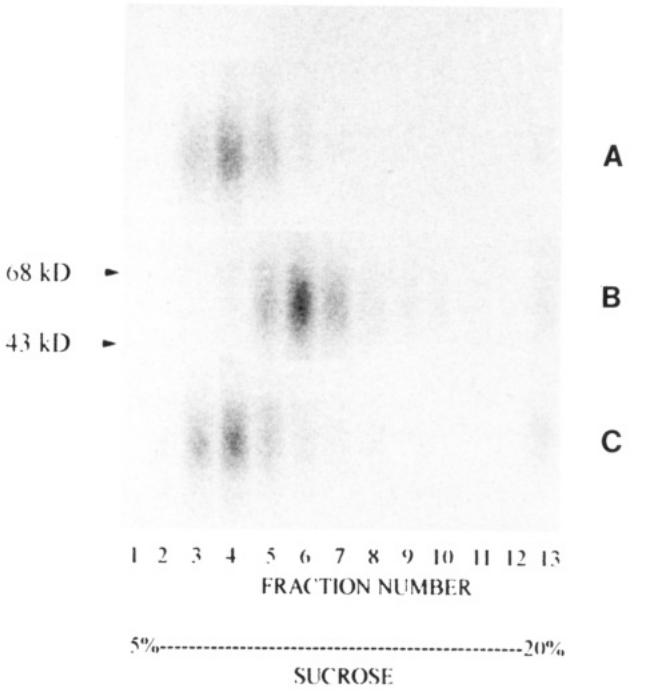

FIGURE 1: Disruption of FPR-G_{i2} complexes by a receptor-mimetic peptide from the predicted third cytoplasmic loop of FPR, C3A (see Table 1). FPR was radioactively labeled with ^{125}I using an agonist analog and is observed as a broad band between the 43- and 68-kDa markers using storage phosphor visualization. The ^{125}I photoaffinity-labeled FPR and reconstituted FPR−G_{i2} complexes were separated by velocity sedimentation in linear sucrose density gradients containing 1% octyl glucoside. Fractions from the density gradients were solubilized in SDS and run on SDS−10% polyacrylamide gels. The gels were dried, and the FPR was quantified after a 20-h exposure to a storage phosphor screen. Panel A shows the FPR sedimentation profile with an average sedimentation coefficient of 4S in the absence of added G_{i2}. Panel B shows the symmetric shift in sedimentation of FPR from 4S to 7S that occurs in the presence of added G_{i2} (400 nM). Panel C shows complete disruption of the 7S complex by 3 mM FPR C3A peptide (see Table 1 for the C3A peptide sequence and the inset of Figure 2 for the location in the receptor sequence). Similar shifts in sedimentation of the reconstituted 7S complex to 4S were observed with other active FPR peptides or with 1−10 μM GTPγS.

loop of FPR, C3A, is shown in panel C of Figure 1. Table 1 shows the peptide sequence and the inset in Figure 2c shows the location of the peptide in our previously proposed model for the transmembrane organization of FPR (Bommakanti et al., 1993). Addition of the C3A peptide produced a more slowly sedimenting form of receptor with a distribution in the gradient (Figure 1C) that was indistinguishable from the G protein-free form of FPR produced by inclusion of GTPγS in the incubation mixture (panel A) (Bommakanti et al., 1992, 1994).

Inhibition of Complex Formation by Receptor-Mimetic Peptides. The effectiveness of C3A and other peptides in dissociating the FPR−G_{i2} complex was assessed by plotting the amount of FPR radioactivity in the more rapidly sedimenting 7S form (Bommakanti et al., 1994) as a function of peptide concentration. Figure 2 shows the dependence of the disruption of the FPR−G_{i2} complex on the concentration of different peptides that mimic regions of FPR adjacent to or within its predicted first (Figure 2a), second (Figure 2b), or third (Figure 2c) cytoplasmic loops and its carboxyl-terminal tail (Figure 2d). In each panel, the percent of the G_{i2}-complexed 7S FPR is plotted as a function of increasing peptide concentration. The concentration at which 50% inhibition (IC_{50}) was achieved for each of the peptides examined is given in Table 1.

The concentration ranges observed for peptides judged to be active in this study were in the ranges accepted as useful to reveal protein−protein interaction for numerous receptor and non receptor systems (Dalman & Neubig, 1991; Hamm et al., 1988; Hamm, 1991; Bommakanti et al., 1993; Krupnick et al., 1994; Kleinberg et al., 1992). We judged peptides as having significant inhibitory activity if they displayed 50% inhibition at 1.5 mM or less and displayed a relatively sharp dose−response curve in which there was at least 50% inhibition over 1.5 log units of concentration. Some peptides with lower activity might overlap functionally active regions but not *fully* include the active sequence. Peptides that include only part of an active sequence might thus be expected to display modest activity, in the low millimolar range, and still provide information that helps to localize the active regions. We termed peptides *inactive* if they displayed less than 10% inhibitory activity at concentrations of 5 mM or above.

First Cytoplasmic Loop. Most work on other GPCR systems (König et al., 1989; Münch et al., 1991) has suggested no role for the first cytoplasmic loop in coupling receptors to G proteins. Recently, Moro et al. (1993) reported that site-specific substitutions in an analogous region of human muscarinic cholinergic receptor (Hm1) resulted in some inhibition of ligand binding and stimulation of phosphatidylinositol turnover. Because site-specific mutagenesis of a protein might produce remote effects resulting in weak inhibition of activity, it was important to examine the analogous region in FPR with synthetic mimetic peptides. Our studies, which examine physical interaction of FPR and G protein, might thus provide an alternative molecular view of such interactions. Interestingly, we found that a peptide corresponding to the first cytoplasmic loop of FPR (Figure 2a) was able to dissociate the reconstituted 7S FPR−G_{i2} complex with an IC_{50} of approximately 100 μM. The first cytoplasmic loop, consequently, was one of the more active peptides investigated (see Table 1) and we conclude that it contributes substantially to the interaction between G protein and FPR.

Second Cytoplasmic Loop. Results generated in other GPCR systems suggests that sequences in or near the predicted second cytoplasmic loop are involved in important functional contacts with G proteins (Hargrave & McDowell, 1992). To attempt to better localize the important sites of interaction, we examined the effect of several FPR-mimetic peptides from this region. The peptide corresponding to sequence of the entire predicted second cytoplasmic loop, C2W, was *the most* active peptide investigated, with an IC_{50} value of approximately 20 μM, as shown in Figure 2b. We confirmed (Bommakanti et al., 1993) that the middle region of the second loop peptide (C2M) was inactive (Figure 2b). Comparing the activity of C2W and C2M suggests that residues 122−126 and/or residues 141−144 (and perhaps residues nearby) may be critical for the activity (inset, Figure 2b). This conclusion is supported by the observation that the two flanking "half-loop" peptides C2B and C2A, overlapping the amino and carboxyl ends of C2W, exhibited IC_{50} values of 1.0 mM and 100 μM, respectively (Figure 2b and Table 1). An (E/D)R dyad is conserved in all GPCR and has been shown, by site-specific mutagenesis, to be required for receptor function (Franke et al., 1990). C2B

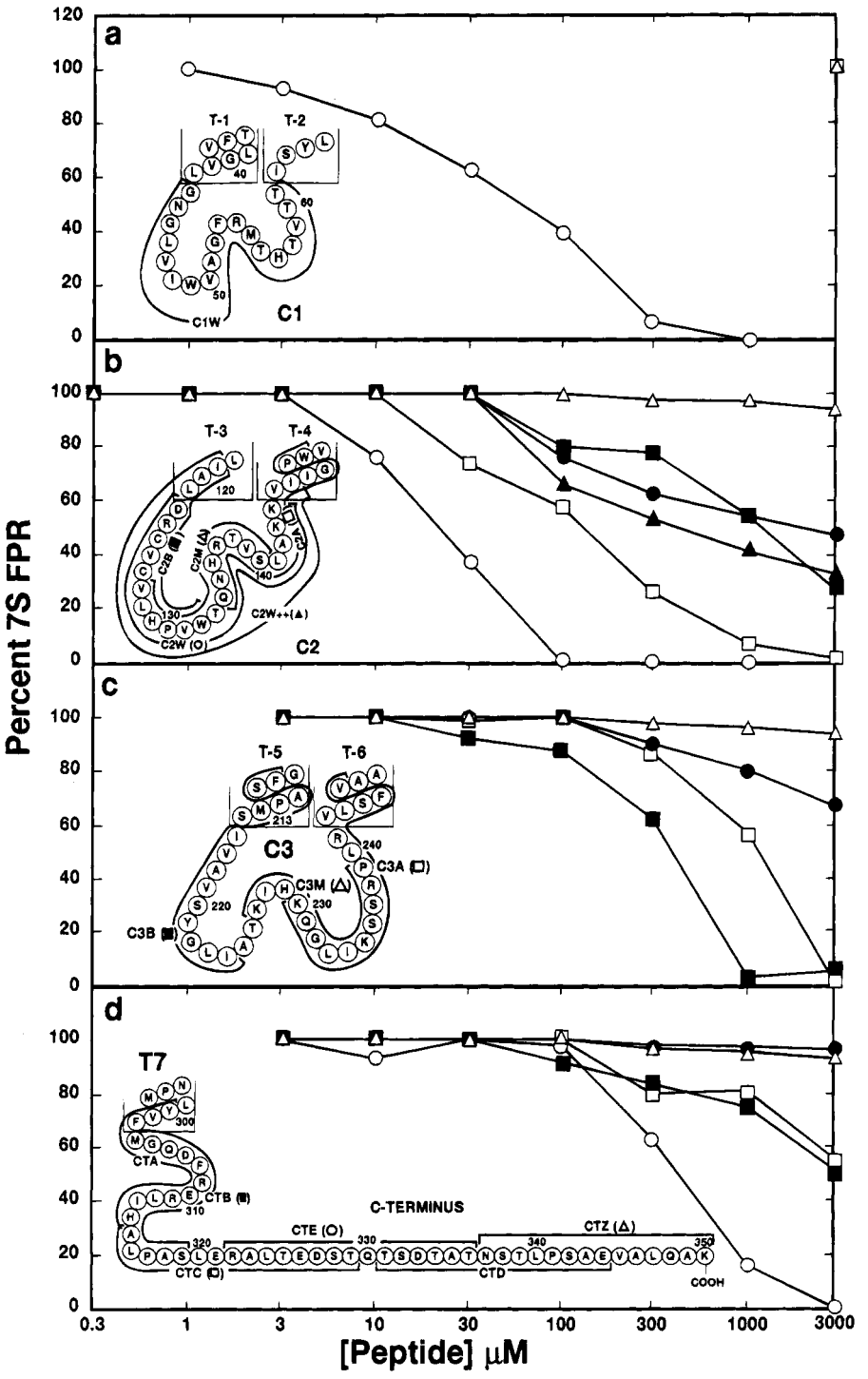

FIGURE 2: Comparison of the dissociation of reconstituted 7S FPR−G_{i2} complexes by receptor-mimetic peptides from the putative cytoplasmic and certain adjacent membrane regions of FPR. The insets show the location of the sequences mimicked by each of the peptides examined in a preliminary folding model of FPR. T-1−T-7 represent portions of the transmembrane helical regions close to the cytoplasmic face of the membrane. The cytoplasmic loops (C1−C3) connect the membrane domains. Regions of the cytoplasmic carboxyl-terminal tails are denoted by CTA−CTZ. The three-digit numbers on the insets indicate the residue numbers in the linear sequence of FPR. The percentage of the total FPR sedimenting with an apparent sedimentation coefficient of 7S was determined as described in the text. Complete inhibition is equivalent to pure 4S form of FPR. The data points shown represent the mean of three different experiments (C1W, 2 experiments). The standard error ranged from 2.3% (C2W at 30 μM) to 8.7% (C2B at 100 μM). (**a**) The peptide used to mimic the FPR domain in the vicinity of the putative first cytoplasmic loop was C1W (43-GNGLVIWVAGFRMTHTVTT-61) (○); control extracellular loop peptide, E1A (85-KAMGGHWPFGWFLCK-99) (△); control extracellular loop peptide, E2A (170-KTGTVACTFNFSPWT-184) (□). (**b**) Peptides used to mimic the FPR domain in the vicinity of the putative second cytoplasmic loop were C2W (122 DRCVCVLHPVWTQNHRTVSLAKK-144) (○), C2W++ (119-IALDRCVCVLHPVWTQNHRTVSLAKKVI-146) (▲), reverse C2W or rC2W (144-KKALSVTRH-NQTWVPHLVCVCRD-122) (●), C2A (134-QNHRTVSLAKKVIIGPW-150) (□), C2B (119-IALDRCVCVLHPVWT-133) (■), and C2M (127-VLHPVWTQNHRTVS-140) (△). (**c**) Peptides used to mimic the FPR domain in the vicinity of the third cytoplasmic loop were C3A (230-KQGLIKSSRPLRVLSFV-246) (□), C3B (210-FSAPMSIVAVSYGLI-224) (■), reverse C3A or rC3A (246-VFSLVRLPRSSKILGQK-230) (●), and C3M (227-KIHKQGLIKSSRP-239) (△). (**d**) Peptides used to mimic FPR sequences of the COOH tail were CTE (322-RALTEDSTQTSDTAT-336) (○), reverse CTE or rCTE (336-TATDSTQTSDETLAR-322) (●), CTC (315-ALPASLERALTEDST-329) (□), CTB (304-MGQDFRERLIHALPAS-319) (■), and CTZ (337-NSTLPSAEVALQAK-350) (△).

and C2W contain this (E/D)R dyad, whereas C2M does not, suggesting that the N-terminal region of C2W, 122-DRCVC-126, may be essential for physical and functional FPR−G_{i2} coupling.

Recent work by Schreiber et al. (1994) suggests that peptide C12R (126-CVLHPVWTQNHR-137) from the second cytoplasmic loop of FPR *was able* to disrupt the reconstituted 7S complex with an IC_{50} of about 300 μM, which is 15-fold less effective than the C2W peptide. The inactive C2M peptide (127-VLHPVWTQNHRTVS-140) differs from the C12R peptide, reported by Schreiber et al., in that it does not contain the amino-terminal cysteine (C126) and has three additional residues at the carboxyl terminus (138-TVS-140). These differences suggest that the addition of a cysteine residue (C126) as well as the (E/D)R dyad (122-DR-123) to the amino terminus of C2M peptide impart activity and/or that the three additional residues on C2M peptide (138-TVS-140) may contribute to improper folding of the peptide, rendering it inactive.

To examine whether the hydrophobic residues of C2B might contribute to FPR−G_{i2} interaction, a longer second loop peptide was synthesized. C2W++ was extended at both ends, compared to C2W, and was found to be 20-fold *less active* than C2W. Therefore, the extreme ends of C2W++ either do not contribute to stability of the interaction or may promote folding of the peptide that interferes with activity. Addition of equimolar C2A and C2B simultaneously to the reconstitution mixture, however, did not reduce the IC_{50} for the 7S disruption below that of the more active C2A alone, indicating that the effects of these two peptides do not appear to be synergistic.

Third Cytoplasmic Loop. We and others (Bommakanti et al., 1993; Schreiber et al., 1994; Prossnitz et al., 1993) have independently shown that the most hydrophilic portion of the third cytoplasmic loop (C3M) appears *not* to be involved in supporting FPR−G protein association. However, if the flanking amino- and carboxyl-terminal regions of the third loop are examined, C3B and C3A, respectively, significant inhibitory activity is observed, displaying IC_{50}s of 0.5 and 1.4 mM, respectively, as shown in Figure 2c. Considering the inactive FPR C3M sequence, the active juxtamembrane regions near the third loop appear to be near residues 240-LRVLSFV-246 of C3A and must include a portion of the FPR C3B (210−224).

Supportive evidence, implicating hydrophobic amino acid receptor domains in G protein activation, have been reported in the case of the β_2-adrenergic receptor (Cheung et al., 1992). These investigators selectively replaced the charged as well as uncharged residues in the amino-terminal end of the third cytoplasmic loop and assessed the ability of the mutant receptors to stimulate adenylyl cyclase and the sensitivity of receptor agonist binding to the nonhydrolyzable GTP analogue Gpp(NH)p. These authors concluded that hydrophobic interactions in this region, presumed to be an extension of the fifth transmembrane domain (analogous to the C3B region of FPR), play a more critical role than ionic or hydrophilic interactions in activating G_s. Recent work by Bluml et al. (1994) is consistent with the N-terminal region of C3 of the muscarinic receptor being in an α-helical conformation and a hydrophobic site of the helix acting as a G protein interaction surface. We found that treatment of neutrophil membranes under conditions known to solubilize peripheral membrane proteins by interfering with ionic interactions (1 M NaCl or 5 mM EDTA) did not dissociate the FPR−G_{i2} complex (Bommakanti et al., 1994), which implies that agonist-occupied FPR−G_{i2} binding involves some specific hydrophobic interfaces. Taken together, these findings suggest that hydrophobic intramembrane extensions of the cytoplasmic domains of agonist-occupied FPR may be accessible to interaction with G protein and may be important for G protein binding to the activated receptor.

Carboxyl-Terminal Tail. Although evidence for involvement of regions in or adjacent to each of the FPR cytoplasmic loops is compelling, a detailed mapping using shorter peptides including and overlapping the sequences in these regions is still required. Such an analysis has been initiated for the carboxyl-terminal tail and the results confirm and extend our previous findings (Bommakanti et al., 1993). The CTE region (Figure 2) appears to be the major structural determinant in the C-terminal region involved in the FPR−G_{i2} interaction. Peptides CTB and CTC covering parts of the CTE peptide or containing upstream sequences were only weakly inhibitory with IC_{50}s of about 3 mM (Figure 2). Interestingly, peptide CTD, containing the other half of CTE, was not effective (data not shown). Schreiber et al. (1994) found that peptides spanning the entire cytoplasmic tail, as well as a fusion protein containing the entire tail region, inhibited fMLF-induced ribosylation of G_i with an IC_{50} of 250 μM peptide concentration. The fusion protein containing the entire tail competed with a $G_{i\alpha}$-specific antibody for binding to G_i protein with an IC_{50} of 20 μM.

The CTB peptide of FPR, corresponding to the juxtamembrane amino terminus of the tail region, had an IC_{50} of more than 3 mM, indicating only marginal activity. The analogous region in rhodopsin and the adrenergic receptors is active. The CTB region in FPR does not contain a cysteine residue, which in some other GPCR is thought to be palmitoylated and to mediate the formation of a fourth cytoplasmic loop (Dohlman et al., 1991). The peptide corresponding to the last 14 residues of the C-terminal tail, CTZ, was also inactive.

Characteristics of Peptide/FPR−G_{i2} Interactions. Our results indicate that any one of several peptides can cause dissociation of FPR from G_{i2}. Thus, we have concluded that the FPR−G_{i2} interaction is made up of multisite contacts whose combined affinities are such that dissociation of the complex occurs with disruption at any particular site, or alternatively, that site-specific disruption is cooperative and has remote effects at other sites. The relative steepness of some of the curves shown in Figure 2 for C2W, C3B, and C3A is similar to that noted in our previous work on CTE (Bommakanti et al., 1993) and, we believe, may be related to such cooperativity.

Application of reverse peptides also provides some information about the nature of the FPR/G_{i2} interaction. We previously found that the reverse CTE sequence (rCTE) had no measurable activity (Bommakanti et al., 1993). Interestingly, certain reverse sequences of the C3A and C2W peptides (rC3A and rC2W) displayed a measurable but much smaller (less than 10%) activity than their corresponding forward sequences. Such results are consistent with a real and measurable low activity of rC3A and rC2W and are supported by observations relating to the activities of the reverse sequence linear peptide epitopes of certain monoclonal antibodies (Burritt et al., 1994). The reverse sequences may be expected to show some activity if the pattern of charges or H bonding is important for the interaction *rather*

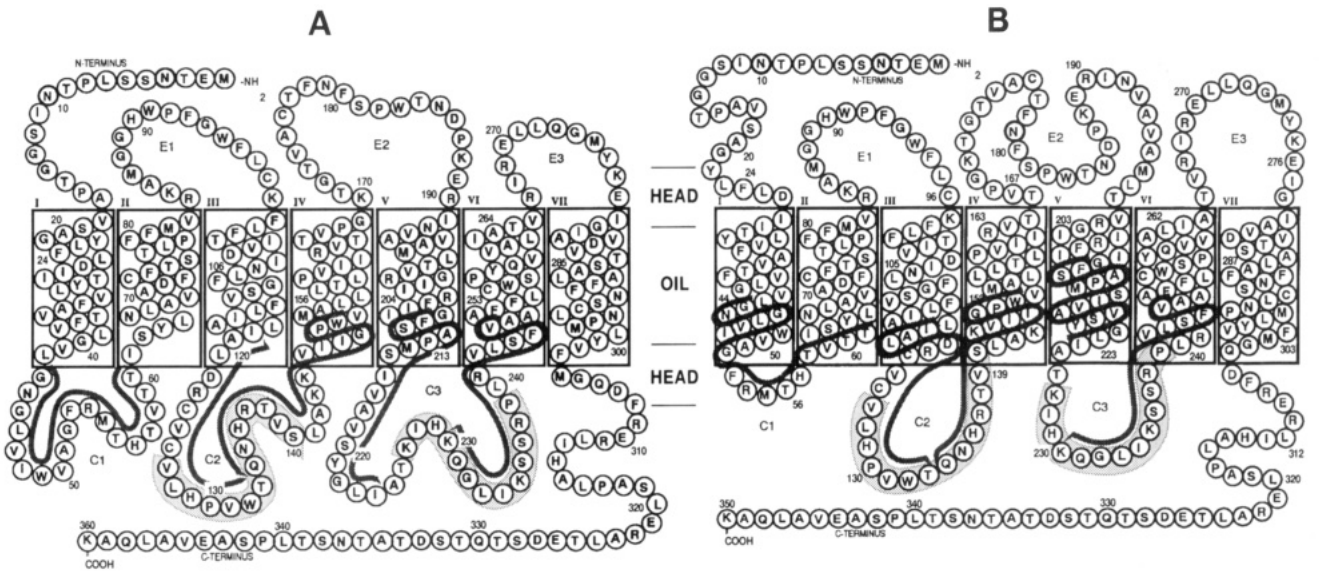

FIGURE 3: Representative models for the transmembrane topology of N-formyl peptide chemoattractant receptor (FPR). Model A, based on hydropathy analysis and sequence alignments of several other GPCR (Hargrave & McDowell, 1992; Probst et al., 1992), has been previously published by us (Bommakanti et al., 1993). Model B is based on more detailed alignments of conserved and nonconserved amino acids of 204 representative GPCR by Baldwin (1993) and is similar to other proposed FPR models (Boulay et al., 1990a; Ye et al., 1993). The regions on the models indicated with heavy lines show some of the peptide sequences which are active in blocking FPR−G_{i2} interactions and where the transmembrane disposition differs greatly between the two models. The cytoplasmic regions indicated by stippled boxes highlight some of the peptide sequences that are inactive in blocking the FPR−G_{i2} interaction.

than the detailed steric features of the bound peptide. Some high-affinity protein−protein interactions are dominated by charge interaction rather than a precise steric fit (Guillet et al., 1993).

The more active peptides near loop 2 have a charge of +3 (C2A and C2W) and have a very modest hydrophilic index (Table 1). The more active loop 3 peptide, C3B, is very hydrophobic (index = −1.12) and has a net charge of 0. The region of C3A that confers activity beyond the inactive C3M has five very hydrophobic residues out of seven (LRVLSFV). These observations suggest that the regions near the second and third cytoplasmic loops probably have different types of interactions in their coupling to G_{i2}. The second loop region may exploit a combination of polar and nonpolar interactions near DRCVC and polar interaction near LAKK, while the third loop may depend much more on hydrophobic domains for G_{i2} binding.

The inhibition of FPR−G_{i2} complex formation was also insensitive to detergent concentration above (1% octyl glucoside) and below (0.5%) the critical micelle concentration (0.8%) for the most hydrophobic peptide, C3B [hydrophilic index (Hopp & Woods, 1981) = −1.12, Table 1), suggesting that the peptide inhibition was not caused by nonspecific hydrophobic binding to hydrophobic regions of the FPR or G protein. Unrelated control peptides, with different and unrelated sequences (e.g., neutrophil $gp91^{phox}$, FPR extracellular domain peptides, peptides derived from the $G_{i2\alpha}$ subunit or actin; as shown in Table I) did not detectably dissociate the 7S complex up to a concentration of 5 mM. We sought to examine peptides having a range of charge, hydrophilicity, and amphiphilicity (see Table 1) similar to those of the active peptides described above. When the net charge, hydrophilic index, and hydrophobic moment for each peptide tested are plotted as a function of IC_{50} (not shown), it is evident that there is no correlation of activity with any of these properties. The peptides that show less or no activation (Table 1) span the full range of these properties and thus serve as important controls indicating that bulk physical properties cannot explain the inhibitory activities observed for the active peptides. Together these observations suggest that peptide-induced dissociation of the 7S FPR−G_{i2} complex was not a result of nonspecific effects.

Structural Models of FPR and the FPR−G_{i2} Complex. Dratz and Hargrave proposed some time ago (Dratz & Hargrave, 1983) that the transmembrane domains of rhodopsin, the visual pigment in the discs of rod outer segments in retina, may form a seven transmembrane helix bundle. This folding pattern has experimental support (Dratz et al., 1985; Schertler et al., 1993) and it is thought that members of the large superfamily of GPCR all contain similar bundles of seven transmembrane helices. Without more detailed direct structural information, however, it is not possible to know the precise transmembrane localization of amino acids spanning the hydrophobic regions of the membrane or the amino acids in the membrane polar head group regions or in the cytoplasmic domains. Thus, models for the folding topography of FPR in the neutrophil plasma membrane must currently depend on hydropathy constraints and analogies to other GPCR for which more detailed information is available. The FPR transmembrane topography model proposed by us (Bommakanti et al., 1993) originally formed the basis for our choice of potentially inhibitory peptides to be tested in the reconstitution assays. This model was based on hydropathy analysis and analogies with models proposed for other GPCR (Dratz & Hargrave, 1983; Hargrave & McDowell, 1992; Probst et al., 1992). Two other models for FPR topography have since been published (Boulay et al., 1990a; Ye et al., 1993) which differ from our model primarily in the disposition of the first and the third predicted cytoplasmic loops.

Recently, a more global consensus model for GPCR was published by Baldwin (1993) which is based on a detailed comparison of conserved and nonconserved residues in the sequences of 204 G protein-coupled receptors that were

chosen to be representative of all known GPCR subfamilies. Other sequences were excluded to avoid biasing the conclusions by subtypes with many known members. The detailed transmembrane helix packing of the Baldwin model had striking success in reproducing the electron density profile of rhodopsin recently observed in 2D crystals in projection (Schertler et al., 1993) and in 3D reconstruction (Unger & Schertler, 1994; Unger et al., 1995). Our original FPR transmembrane topology model is shown in Figure 3, panel A (termed model A) and is compared with model B after Baldwin (Figure 3, panel B). The two other FPR models (Boulay et al., 1990a; Ye et al., 1993) are rather similar to model B in Figure 3. Models A and B are similar in the predicted disposition of the starting point of the C-terminal tail. The largest differences between the two models are that model B has significantly shorter C1, C2, and C3 loops and that two arginine residues (R201 and R205) of transmembrane helix 5 and one aspartic acid (D26) of transmembrane helix 1 are placed in the polar/hydrophobic interface in model B. The consensus Baldwin model predicts that *both* the C2 and C3 cytoplasmic loops very closely match the totally inactive C2M and C3M peptides (shaded in Figure 3) whereas active C2A, C2B, C3A, and C3B peptides extend well into the transmembrane region. The fact that the most hydrophilic midregions of the second and third cytoplasmic loops are totally inactive in blocking FPR−G_{i2} interaction while some more hydrophobic regions are active suggests the G protein recognition of FPR may not involve the midregions of the cytoplasmic loops. Conceptually, this result could be achieved by the model for the receptor−G protein complex proposed by Dratz et al. (1993), where part of the G_α subunit penetrates into the transmembrane region. Alternatively, these more hydrophilic regions might contribute to G protein activation by imposing a negative or repulsive energy on the complex which would favor conformational changes and remain undetectable in our assay system.

In summary, our work has resulted in novel observations that may have significant implications for GPCR structure and functions. First, the interaction of agonist-occupied FPR with G_{i2} must be mediated by extensive protein−protein contact involving interaction of *all* the cytoplasmic subdomains of FPR, including the putative first cytoplasmic loop. Second, the agonist-occupied FPR−G_{i2} interaction may well involve interdigitation of the two proteins, which is likely to include interaction of hydrophobic regions of both proteins. Third, if interpreted in the light of a current model of receptor structure, the most hydrophilic central regions of the second and third cytoplasmic loops appear *not* to be important for stabilizing the interaction between FPR and G protein, while membrane regions of both loops are important. Last, at least six distinct regions of the receptor are involved in the interaction with G_{i2} (that we designate C1W, C2A, C2B, C3A, C3B, and CTE). These new ramifications of the agonist-occupied FPR−G_{i2} interaction may provide a more detailed working model to guide the design of experiments to bring us closer to a better understanding of the molecular basis of neutrophil chemotaxis and G protein-coupled signal transduction in general.

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