The human formyl peptide receptor (FPR) is a prototypical G protein-coupled receptor, but little is known about quantitative aspects of FPR-G protein coupling. To address this issue, we fused the FPR to $G_{i1}$, $G_{i2}$, and $G_{i3}$ and expressed the fusion proteins in SF9 insect cells. Fusion of a receptor to $G_i$ ensures a defined 1:1 stoichiometry of the signaling partners. By analyzing high affinity agonist binding, the kinetics of agonist- and inverse agonist-regulated guanosine 5'-O-(3-thiotriophosphate) (GTPγS) binding and GTP hydrolysis and photolabeling of $G_i$, we demonstrate highly efficient coupling of the FPR to $G_{i1}$, $G_{i2}$, and $G_{i3}$ without cross-talk of the receptor to insect cell $G$ proteins. The FPR displayed high constitutive activity when coupled to all three $G_i$ isoforms. The $K_a$ values of high affinity agonist binding were ~100-fold lower than the $EC_{50}$ (concentration that gives half-maximal stimulation) values of agonist for GTPase activation. Based on the $B_{max}$ values of agonist saturation binding and ligand-regulated GTPγS binding, it was previously proposed that the FPR activates $G$ proteins catalytically, i.e. one FPR activates several $G$ proteins. Analysis of agonist saturation binding, ligand-regulated GTPγS saturation binding and quantitative immunoblotting with membranes expressing FPR-$G_i$ fusion proteins and nonfused FPR now reveals that FPR agonist binding greatly underestimates the actual FPR expression level. Our data show the following: (i) the FPR couples to $G_{i1}$, $G_{i2}$, and $G_{i3}$ with similar efficiency; (ii) the FPR can exist in a state of low agonist affinity that couples efficiently to $G$ proteins; and (iii) in contrast to the previously held view, the FPR appears to activate $G$ proteins linearly and not catalytically.

Most intercellular signal molecules exert their effects through GPCRs3 that couple to heterotrimeric G proteins, which in turn regulate the activity of effector systems (1-3).

The extended ternary complex model of receptor activation assumes that GPCRs exist either in an inactive “R” state or an active “R*” state (4-6). GPCRs can isomerize from R to R* spontaneously, and this process is referred to as constitutive activity. Receptor agonists stabilize the R* state and increase basal $G$ protein activity, whereas inverse agonists stabilize the R state and reduce basal $G$ protein activity (4, 6, 7).

The FPR is expressed predominantly in phagocytic cells and plays a crucial role in host defense against bacterial infections (8-10). The FPR couples to the pertussis toxin-sensitive $G$ proteins $G_{i2}$ and $G_{i3}$ (11-13) and, via released G protein βγ-subunits, mediates activation of phospholipase C-β2 (14, 15) and phosphatidylinositol-3-kinase (16-18). Consequently, phagocyte functions such as chemotaxis, lysosomal enzyme release, and superoxide radical formation are activated (8, 19-21). Based on the comparison of $B_{max}$ values of agonist saturation binding and ligand-regulated GTPγS binding, it was proposed that the FPR activates $G$ proteins catalytically, i.e. one FPR activates several $G$ proteins (22-25).

Several questions regarding the quantitative aspects of FPR-$G_i$ coupling are still unresolved. Specifically, it is unclear why the $K_a$ value for high affinity $[^{3}H]fMLF$ binding is ~3 nM, whereas the $EC_{50}$ value of fMLF at activating $G_i$ proteins in terms of GTP hydrolysis, AA-GTP labeling, and GTPγS binding is much higher (~0.1-1 μM) (8, 12, 25, 26). Similarly, it is unknown why fMLF is much more potent at activating chemotaxis than lysosomal enzyme release and superoxide radical formation (8, 19-21). Furthermore, it is not clear whether the FPR couples differentially to $G_i$ isoforms.

This latter question is intriguing in view of several findings. First, phagocytes express $G_{i2}$ at a much higher level than $G_{i3}$ and $G_{i1}$ is not expressed at all (11, 13, 27, 28). Second, certain GPCRs, including the receptors for the chemottractant interleukin-8, differ substantially in their coupling efficiency to various $G$ isoforms (29-34). Third, data obtained with the $G_{i2}$ knock-out mouse suggest that this $G$ protein has unique functions in signal transduction (35, 36).

Perhaps most importantly, $G_{i2}$ has a lower GDP affinity than $G_{i1}$ and $G_{i3}$ (37, 38). For $G_i$ isoforms, it has been shown that the GDP affinity of $G_o$ has a substantial impact on the efficiency of receptor-$G$ protein coupling. Particularly, $G_{i1L}$ (the long splice variant of the α-subunit of the stimulatory $G$ protein of adenyl cyclase $G_s$) has a lower GDP affinity than $G_{i2}$ (the short splice variant) (7, 39). Thus, the GPCR activation energy required for releasing GDP from $G_{i1L}$ is lower than the corresponding activation energy needed for GDP release from $G_{i2}$. Accordingly, the βγAR catalyzes GDP release from $G_{i2L}$ more readily than from $G_{i3L}$. Experimentally, this results in increased efficacy and potency of partial agonists and increased efficacy of inverse agonists when the βγAR is coupled to $G_{i2L}$ as compared with the corresponding ligand properties when coupling of the βAR to $G_{i3L}$ is considered (7). In other

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The abbreviations used are: GPCR, G protein-coupled receptor; AA-GTP, (α-32P)GTP azidoanilide; β2AR, β2-adrenergic receptor; Boc-FLMLF, N-t-butoxycarbonyl-L-phenylalaninyl-L-leucyl-L-phenylalaninyl-L-lyeucl-L-phenylalaninle; CsH, cyclosporin H; DHA, dihydroalprenolol; fMLF, N-formyl-L-methionyl-L-leucyl-L-phenylalaninle; IMW, N-formyl-L-methionyl-L-tryptophan; FPR, formyl peptide receptor; GTPγS, guanosine 5'-O-(3-thiotriophosphate); MLF, L-methionyl-L-leucyl-L-phenylalaninle; PAGE, polyacrylamide gel electrophoresis; POMC, pro-opiomelanocortin; h, peak pair(s).
words, Gα1a conveys to the βγAR the properties of a constitutively active GPCR. Intriguingly, when coupled to Gα12, the FPR is constitutively active as well as assessed by strong inhibitory effects of the inverse agonist CsH on the high basal GTPγS binding (25). Taken together, all these findings raise the question of the impact of the different Gα isoforms on constitutive activity of the FPR.

The aim of our present study was to quantitatively analyze FPR coupling to the three Gα isoforms. To achieve this aim, we fused the FPR to Gαt, Gαz2, and Gαz3 and expressed the fusion proteins in SF9 cells. Fusion of GPCR to Gα ensures a defined 1:1 stoichiometry and efficient coupling of the signaling partners and allows for the sensitive detection of differences in the coupling of a given GPCR to different G proteins (7, 40–43).

**Experimental Procedures**

Materials—The cDNAs of Gαt, Gαz2, and Gαz3 in pGM-2 were kindly provided by Dr. R. Reed (Howard Hughes Medical Institute, Johns Hopkins University, Baltimore, MD) (44). The baculovirus encoding Gαz3 was kindly provided by Dr. A. G. Gilman (Department of Pharmacology, University of Southwestern Pennsylvania, Pittsburgh, PA) (45). The antibody recognizing the C terminus of Gαt (AS 86) (46) was generously provided by Drs. B. Nürnberg and G. Schultz (Institut für Pharmakologie, Freie Universität Berlin, Berlin, Germany). The anti-FLAG Ig (M1 monoclonal antibody) was from Sigma. The antibody recognizing the C terminus of Gαz2 was from Calbiochem. The anti-His Ig was from CLONTECH. [γ-32P]GTP (6000 Ci/mmol), [α-32P]GTP (3000 Ci/mmol), [35S]GTP-γS (1100 Ci/mmol), and [3H]GTP (56 Ci/mmol) were from Amersham Pharmacia Biotech. The cDNAs of Giα1, Giα2, and Giα3 in Giα1, Giα2, and Giα3 expression vector pVL 1392, the cDNAs encoding fusion proteins in pGEM-3Z SacII-XbaI at the 5′-end of the respective Gα isoform were digested with 5′-end extension with HindIII at the 5′-end of the SF region, blunted with DNA Polymerase I (Klenow fragment), and then digested with XbaI at the 3′-end of Gα. Digested fusion protein DNAs were then transferred into pVL 1392 that had been digested with HindIII, blunted with Klenow fragment, and subsequently digested with XbaI. The DNA for the βγAR-αf fusion protein (used as immunoblotting standard for the determination of Gαz3 expression) was prepared by overlap extension PCR analogous to the FPR-Gα fusion protein DNAs. PCR-generated sequences were confirmed by enzymatic sequencing.

**Generation of Recombinant Baculoviruses, Cell Culture, and Membrane Preparation**—Generation of baculoviruses, cell culture, and membrane preparation were performed exactly as described (25, 41). SF9 cells were co-infected with recombinant baculoviruses encoding nonfused FPR, Gαt, and G protein βγγ complex or fusion proteins plus G protein βγγ complex.

**Analysis of FPR-Gα Fusion Proteins by Agonist Saturation Binding, GTPγS Binding, and GTPase Activity—**[H]MLF saturation binding, GTPγS saturation binding, and time course of GTPγS binding in SF9 membranes expressing FPR-Gα fusion proteins plus βγγ complex were performed exactly as described for SF9 membranes expressing nonfused FPR, Gαt, and βγγ complex (25). Steady-state GTPase activity with different substrate concentrations was determined as described for SF9 membranes expressing βAR-Giα1 fusion protein except that the MgCl2 concentration was 5 mM instead of 1 mM (41).

**Analysis of FPR-Gα Fusion Proteins on the Protein Level—**SF9 membranes were analyzed by immunoblotting using the monoclonal M1 antibody, which recognizes the N-terminal FLAG epitope of the FPR and βAR (25, 41), anti-His Ig, which recognizes the C-terminal His6 epitope of the FPR and βAR (25, 41), anti-Giα1,2 Ig, and anti-Giα3 Ig. The M1 antibody was used at a dilution of 1:1000. The other antibodies were used at a dilution of 1:500. SDS-PAGE and immunoblotting were performed exactly as described (25).

**Analysis of FPR-Gα Fusion Protein Expression on the mRNA Level**—mRNA from SF9 cells infected with recombinant baculoviruses was isolated with the RNeasy kit from Qiagen and treated with RNase-free DNase. mRNA was reverse-transcribed using the First Strand cDNA synthesis kit from Amersham Pharmacia Biotech. The cDNAs of Gαt, Gαz2, and Gαz3 in FPR-Gα fusion proteins were amplified using appropriate primer pairs. PCR products were digested with various restriction enzymes, separated by electrophoresis on gels containing 2% (w/v) agarose, and visualized by ethidium bromide staining.

**Miscellaneous**—Protein was determined using the Bio-Rad DC protein assay kit. Data were analyzed by nonlinear regression, using the Prism program.

**Results**

**Analysis of FPR-Gα Fusion Protein Expression on the Protein and mRNA Level**—The human FPR expressed in SF9 cells has a molecular mass of 40 kDa (25), and the molecular mass of Gα proteins is 40–41 kDa (11, 13). Thus, the expected molecular mass of FPR-Gα fusion proteins is 80–81 kDa. Indeed, the M1 antibody, which recognizes the FLAG epitope attached to the N terminus of the FPR (25) detected antigens of the appropriate mass in membranes prepared from baculovirus-infected SF9 cells (Fig. 1A). The anti-Gα1,2 Ig detected the FPR-Gα1 and FPR-Gα2 fusion proteins but not FPR-Gα3 (Fig. 1B). In contrast, the anti-Gα3 Ig reacted with the FPR-Gα3 fusion protein but not with FPR-Gα1 or FPR-Gα2 (Fig. 1C). There was no indication for proteolytic degradation of fusion proteins. Because the anti-Gα1,2 Ig cannot discriminate between Gα1 and Gα2 (Fig. 1B), we performed reverse transcriptase-PCR analysis on mRNA from SF9 cells infected with the FPR-Gα1 and FPR-Gα2 baculoviruses to differentiate between the two Gα isoforms. Gα1 has a unique SacI site at position 382, and Gα3 has a unique BamHI site at position 659 (Fig. 1D). The Gα1 portions of fusion proteins were amplified by PCR and digested. As expected, one fragment was amplified with Gα3 cDNA (expected 382- and 711-bp fragment with Gα3, whereas Gα1 cDNA was not cut. In contrast, digestion with BamHI yielded the expected 443- and 653-bp fragments with Gα3, whereas Gα1 cDNA was not cut. Taken together, the immunoblotting and reverse transcriptase-PCR data document the specific expression of FPR-Gα1, FPR-Gα2, and FPR-Gα3 fusion proteins in SF9 cell membranes.
**[H]MLF Saturation Studies**—Fig. 2 (A and B) shows representative [H]MLF saturation binding curves for FPR-G, and Table I summarizes the data for all three fusion proteins. For comparison, the [H]MLF binding data for nonfused FPR co-expressed with G, are included as well in Table I. FPR-G fusion proteins bound the agonist [H]MLF (0.2–30 nM) according to single-site saturation curves. The K and B max values of [H]MLF binding to FPR-G, FPR-G, and FPR-G were similar to each other and comparable with the values for the co-expression system. An increase of the [H]MLF concentration up to 300 nM in the fusion protein and co-expression systems did not further increase B max of agonist binding as compared with a [H]MLF concentration of 30 nM (data not shown), indicating that a possible low affinity agonist-binding site of the FPR cannot be unmasked by means of agonist saturation binding. Binding of GTPγS to Gα un couples GPCRs from G proteins and reduces high affinity agonist binding (7, 25, 48). By analogy to nonfused FPR co-expressed with G, GTPγS substantially reduced the B max of [H]MLF binding to FPR-G, fusion proteins and, to a variable extent, increased the K for [H]MLF.

**Kinetics of GTPγS Binding**—Ligand-regulated GTPγS binding to G proteins is a sensitive method for studying GPCR-G protein coupling, specifically for analyzing the time course of G protein activation and the stoichiometry of coupling (22, 25, 49). Fig. 2C shows a representative time course experiment for the GTPγS binding to FPR-G, and Fig. 2D shows a representative GTPγS saturation binding experiment for FPR-G, Table II summarizes the GTPγS binding data for all three fusion proteins. For comparison, the GTPγS binding data for the FPR co-expressed with G, are included in Table II as well.

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**Fig. 1. Analysis of the expression of FPR-G, fusion proteins in Sf9 cells.** A–C, membranes from Sf9 cells expressing FPR-G, fusion proteins at ~1.2–1.4 pmol/mg as assessed by [H]MLF saturation binding were prepared, separated by SDS-PAGE, and probed with anti-FLAG Ig (A), anti-G, Ig (B), and anti-G, Ig (C) as described under “Experimental Procedures.” Numbers on the left indicate molecular masses of marker proteins. Shown are the horseradish peroxidase-reacted nitrocellulose membranes of gels containing 10% (w/v) acrylamide. D, mRNA from Sf9 cells infected with FPR-G, and FPR-G, baculovirus, respectively, was isolated and reverse-transcribed as described under “Experimental Procedures.” The G, portions of fusion proteins were amplified by PCR and digested with SacI or BamHI. Digested DNA was separated on gels containing 2% (w/v) agarose. The lanes represent the 500- and 1000-bp standards. The scheme on the left shows the relative positions of the SacI site in G, and the BamHI site in G,.

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**Fig. 2. Functional analysis of the FPR-G, fusion protein.** Sf9 membranes expressing FPR-G, at 1.0–2.2 pmol/mg as assessed by [H]MLF saturation binding were subjected to various analyses as described under “Experimental Procedures.” A and B, [H]MLF saturation binding. Reaction mixtures contained Sf9 membranes, [H]MLF at the concentrations indicated on the abscissa and solvent (control) or GTPγS (10 μM). A shows the specific binding in the absence and presence of GTPγS, i.e. the binding not competed for by 10 μM unlabeled GTPγS. B shows the difference between [H]MLF binding in the absence and presence of 10 μM GTPγS. C, time course of GTPγS binding. Reaction mixtures contained Sf9 membranes, 1 nM [32P]GTPγS plus 9 nM unlabeled GTPγS, 1 μM GDP, and solvent (basal), GTPγS (10 μM), or CsH (10 μM). Reactions were stopped at the time points indicated on the abscissa. D, effects of D4F and CsH on GTPγS saturation binding. Reaction mixtures contained Sf9 membranes, 0.1–1 nM [32P]GTPγS plus unlabeled GTPγS at different concentrations to give final ligand concentrations of 0.1–10 nM, 1 μM GDP, and solvent (basal), GTPγS (10 μM), or CsH (10 μM). For each GTPγS concentration, the basal GTPγS binding was subtracted from the GTPγS binding observed in the presence of fMLF to calculate the increase in GTPγS binding caused by fMLF. From each basal GTPγS concentration, the GTPγS binding value observed in the presence of CsH was subtracted to obtain the decrease of GTPγS binding caused by CsH. The dotted line is the extrapolation of basal GTPγS binding. E, concentration-response curves for various FPR ligands on steady-state GTPase activity. Reaction mixtures contained Sf9 membranes, 100 nM γ[32P]GTP, and solvent (2% v/v MeSO) or ligands at various concentrations. F, kinetics of steady-state GTP hydrolysis. Reaction mixtures contained Sf9 membranes, 30 nM-1.5 μM γ[32P]GTP and solvent (2% v/v MeSO) or ligands at various concentrations. As reported previously for the FPR co-expressed with G, in Sf9 cells (25), the FPR fused to G, displayed high constitutive activity as assessed by the strong inhibitory effect of the inverse agonist CsH on GTPγS binding. CsH also displayed strong inhibitory effects at the FPR fused to G, and G,.

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As reported previously for the FPR co-expressed with G, in Sf9 cells (25), the FPR fused to G, displayed high constitutive activity as assessed by the strong inhibitory effect of the inverse agonist CsH on GTPγS binding. CsH also displayed strong inhibitory effects at the FPR fused to G, and G,.
### Table I

| FPR-Gα1 | FPR-Gα2 | FPR-GαS | FPR + GαS |
|---------|---------|---------|-----------|
| GTP-s (pmol/mg) | 1.00 ± 0.02 | 1.00 ± 0.02 | 1.00 ± 0.02 |
| GTP-s (pmol/mg) | 1.00 ± 0.02 | 1.00 ± 0.02 | 1.00 ± 0.02 |

### Table II

Effects of fMLF and CsH on [35S]GTPγS binding in S9 membranes expressing FPR-Gα fusion proteins and nonfused FPR with Gαiε binding saturation and time course

Membranes from S9 cells expressing FPR-Gα fusion proteins were prepared. For saturation binding experiments, membranes were incubated for 60 min in the presence of 0.1–1 nM [35S]GTPγS plus unlabeled GTPγS at different concentrations to give final ligand concentrations of 0.1–10 nM, 1 μM GDP, and solvent (basal), MLF (10 μM), or CsH (10 μM). Nonspecific binding was determined in the presence of 10 μM unlabeled GTPγS. For each GTPγS concentration, the basal GTPγS binding value was subtracted from the GTPγS binding value observed in the presence of MLF to calculate the increase in GTPγS binding caused by fMLF. From each basal GTPγS binding value, the GTPγS binding value observed in the presence of CsH was subtracted to obtain the decrease of GTPγS binding caused by CsH. Saturation binding data were analyzed for best fit to single-site and two-site saturation curves. All data were best fit to a monophasic saturation curve. Data shown are the means ± S.D. of three experiments with different membrane preparations performed in triplicates. The data for the nonfused FPR co-expressed with Gαε were taken from Ref. 25.

| FPR-Gα1 | FPR-Gα2 | FPR-GαS | FPR + GαS |
|---------|---------|---------|-----------|
| GTP-s (pmol/mg) | 1.00 ± 0.02 | 1.00 ± 0.02 | 1.00 ± 0.02 |
| GTP-s (pmol/mg) | 1.00 ± 0.02 | 1.00 ± 0.02 | 1.00 ± 0.02 |

(B_{max total}) is the difference between maximum GTPγS binding stimulated by MLF and minimum GTPγS binding inhibited by CsH. As is evident from the comparison of B_{max} values for [3H]MLF binding and maximum values of GTPγS binding (compare Tables I and II and Fig. 2, A, B, and D), B_{max total} of GTPγS binding surpasses B_{max} of [3H]MLF binding by 4.7-fold for FPR-Gα1, by 5.9-fold for FPR-Gα2, and by 2.5-fold for FPR-GαS.

Resolution of the Discrepancies between the B_{max} of [3H]MLF Binding and Ligand-regulated GTPγS Binding—Two explanations for the discrepancies between the B_{max} values of [3H]MLF binding and ligand-regulated GTPγS binding have to be considered. First, the fused FPR could interact not only with its fused Gαε partner but also with G proteins of the host cell. Indeed, cross-talk of fused G protein-coupled GPCR to endogenous G proteins occurs in some mammalian expression systems (50, 51). Second, the FPR could still couple only to its fused Gαε partner but may do so in a state of low agonist affinity. This low agonist affinity state may not be detected in the [3H]MLF binding assay but in the GTPγS binding assay because the receptor promotes binding of GTPγS to the G protein. The efficient coupling of GPCRs to G proteins in a state of low agonist affinity had already been postulated in previous studies (48, 52).

To address the first explanation, we visualized FPR-activated G proteins by photoaffinity labeling with AA-GTP (12, 13, 47). These studies were performed in the presence of NaCl (see “Experimental Procedures”) to facilitate detection of agonist responses and to suppress agonist-independent labeling of G proteins as the result of high constitutive receptor activity (25). fMLF efficiently stimulated the incorporation of AA-GTP into all three FPR-Gα fusion proteins (Fig. 3). However, fMLF did not stimulate the incorporation of AA-GTP into 40–45-kDa proteins, i.e. endogenous insect cell G proteins (46). These data rule out the possibility of cross-talk of the fused FPR to endogenous G proteins of S9 cells. In agreement with the data regarding fused FPR, nonfused FPR also does not couple to insect cell G proteins (25, 53).

To address the second explanation, we determined the expression of FPR-Gα fusion proteins by a method that is independent of radioligand binding. Specifically, we took advantage of the FLAG and His epitopes that are located at the N and C termini, respectively, of the GPCR portions of fusion proteins (see “Experimental Procedures”) (41, 43). The previously characterized βAR-Gα1 fusion protein can be used as standard to assess the expression level of other proteins bearing the same epitopes because the expression level of βAR-Gα1 can be unequivocally determined by receptor antagonist saturation binding (41, 43). Fig. 4A shows that the immunoreactivity of FPR-Gα1 expressed at 1.4 pmol/mg ([3H]MLF saturation binding) was even higher than the immunoreactivity of βAR-Gα1 expressed at 8.6 pmol/mg ([3H]DAH saturation binding) using the anti-His5 Ig. The signals with FPR-Gα2 (Fig. 4A) and FPR-GαS (data not shown) expressed at 1.0 pmol/mg each were slightly smaller than the signals obtained with βAR-Gα1 expressed at 8.6 pmol/mg. By using the anti-FLAG Ig, the immunoreactivity with βAR-Gα1 expressed at 8.6 pmol/mg was moderately higher than with FPR-Gα1 expressed at 1.4 pmol/mg. The difference in sensitivity between the anti-FLAG Ig and anti-His5 Ig could be due to the fact that the FPR is heavily glycosylated at the extreme N terminus (25, 53, 54) and that this glycosylation could interfere with the recognition of the N-terminal FLAG epitope by the M1 antibody.

To answer the question of whether [3H]MLF saturation binding also underestimates the expression level of nonfused FPR, we compared the immunoreactivity of membranes expressing the βAR or the FPR plus Gαε plus βγ5 complex using the anti-FLAG Ig. Fig. 4C shows that the FPR runs as a much more diffuse band in SDS-PAGE than the βAR, indicating that the FPR is more heavily glycosylated than the βAR. Despite this difference in glycosylation pattern of the two GPCRs, it is evident that the immunoreactivity of the βAR expressed at 3.9 pmol/mg ([3H]DAH saturation binding) is comparable with the immunoreactivity of the FPR expressed at 1.1 pmol/mg ([3H]MLF saturation binding). Unfortunately, we...
could not visualize nonfused GPCRs with the H5m Ig. Apparently, the H5m Ig cannot efficiently recognize the H5m tag when directly located at the C terminus of the antigens studied.

Taken together, the quantitative immunoblotting studies demonstrate that [3H]fMLF saturation binding underestimates the actual expression level of the fused and nonfused FPR by a factor of 4–8, depending on which antibody is used for the detection of the GPCRs. This factor agrees well with the factors obtained in the [3H]fMLF and GTPγS saturation binding studies (Tables I and II). The similarity of the fusion protein expression levels as determined by immunoblotting and GTPγS saturation binding also implies that the majority, if not all, of the expressed fusion protein molecules are functionally active. Of interest, by comparing the Bmax values of agonist and antagonist binding at the adenosine A1 receptor co-expressed with, or fused to, Gα proteins, it was also shown that agonist binding underestimates GPCR expression level by ~3–6-fold (55). Unfortunately, an antagonist radioligand for the FPR is not available, but the combination of immunoblotting and ligand-regulated GTPγS binding can compensate for this deficiency.

We also assessed the potency of fMLF at activating the fused Gα partner. If the FPR in a state of low agonist affinity does indeed couple to Gα proteins, then the EC50 values for fMLF in functional assays should be considerably higher than the Kd values in the agonist binding studies. In fact, at all three fusion proteins, fMLP activated GTP hydrolysis with EC50 values that are ~80–125-fold higher than the Kd values for high affinity [3H]fMLF binding (compare Fig. 2 (A, B, and E) and Tables I and III). A similar discrepancy between agonist affinity and agonist potency was reported for nonfused FPR expressed in HL-60 leukemia cells and Sf9 cells (25, 26).

**GTP Turnover Measurements**—The defined 1:1 stoichiometry of GPCR and Gα in fusion proteins allows determination of ligand-regulated GTP turnover in a membrane system (41, 42). Specifically, GTPase activities are divided by Bmax values of receptor antagonist saturation binding to calculate ligand-regulated GTP turnover in fusion proteins (41, 42). However, because agonist saturation binding largely underestimates the actual expression level of FPR-Gα fusion proteins (Tables I and II and Figs. 2, A, B, and D, and 4), we divided GTPase activities by the Bmax values of ligand-regulated GTPγS binding. Fig. 2F shows a representative experiment for the kinetics of ligand-regulated GTP turnover at FPR-Gα, and Table IV summarizes the data for all three fusion proteins. The Kn values of fMLF-stimulated GTP hydrolysis for FPR-Gα fusion proteins were similar to each other as were the Kd values for CsH-inhibited GTP hydrolysis. The Vmax values of ligand-regulated GTP turnover were similar for the three FPR-Gα fusion proteins. The kinetic parameters of the GTPase of FPR-Gα fusion proteins are also similar to the corresponding parameters of an α2-adrenoceptor-Gα fusion protein (42).

**Determination of Agonist Efficacies and Potencies**—The data obtained with the full agonist fMLF and the full inverse agonist CsH in GTPγS binding and GTPase studies (Tables II–IV) point to high constitutive activity of the FPR coupled to all Gα isoforms. We wished to explore the hypothesis that there are, nonetheless, subtle differences in the constitutive activity of the FPR coupled to Gα isoforms that can only be detected with partial agonists. According to the extended ternary complex model, an increase in constitutive activity is accompanied by an increase in partial agonist efficacy and potency (7, 56, 57). The GTPase assay is particularly suitable for determination of ligand efficacies and potencies because it monitors, unlike the GTPγS binding assay, receptor-G protein coupling at steady state (7, 41). Moreover, the GTPase assay has already successfully been used to dissect differences in the constitutive activity of a given receptor coupled to different Gα isoforms (7).

We analyzed a panel of peptides and identified fMW as
Membranes expressing FPR-G₁α fusion proteins were incubated in the presence of 100 nM [γ-32P]GTP (0.3 μCi/tube), solvent (2% v/v Me₂SO) or ligands at the following concentrations: fMLF (1 nM to 100 μM), fMW (1 nM to 1 μM), MLF (1 nM to 20 μM), CSF (1 nM to 20 μM), and BocFLFLF (1 nM to 20 μM). Concentration-response curves were best fit to sigmoidal saturation curves. EC₅₀ values for agonists and IC₅₀ values for CSF, corresponding to ligand effects, were obtained from fitted sigmoidal saturation curves. The efficacies of ligands were calculated by dividing the maximal ligand-stimulated (or CsH-inhibited) GTPase activity by the maximal fMLF-stimulated GTPase activity. The maximum effect of fMLF was set 1.00. The maximum ligand effects were derived from sigmoidal saturation curves, too. Data shown are the means ± S.D. of three experiments with different membrane preparations performed in triplicates. NA, not applicable (because of the minimal effects of BocFLFLF, precise potencies could not be calculated).

| Ligand    | Efficacy | Potency μM | Efficacy | Potency μM | Efficacy |
|-----------|----------|------------|----------|------------|----------|
| fMLF      | 1.00     | 0.56 ± 0.25| 1.00     | 0.36 ± 0.27| 1.00     |
| fMW       | 0.66 ± 0.14| 8.46 ± 1.16| 0.77 ± 0.05| 8.90 ± 4.23| 0.86 ± 0.03| 4.73 ± 2.41|
| MLF       | 0.44 ± 0.07| 9.35 ± 3.24| 0.45 ± 0.08| 4.97 ± 2.44| 0.53 ± 0.09| 2.21 ± 1.09|
| CsH       | 0.50 ± 0.05| 0.30 ± 0.27| 0.65 ± 0.11| 1.02 ± 0.35| 0.56 ± 0.23| 0.39 ± 0.18|
| BocFLFLF  | 0.01 ± 0.01| NA         | 0.07 ± 0.03| NA         | 0.05 ± 0.02| NA         |

**TABLE IV**

**Kinetics of steady-state GTP hydrolysis in Sf9 membranes expressing FPR-G₁α fusion proteins**

Membranes expressing FPR-G₁α fusion proteins were incubated in the presence of 30 nM to 1.5 μM [γ-32P]GTP (0.5 μCi/tube) and solvent (basal), fMLF (10 μM), or CsH (10 μM). For each GTP concentration, the basal GTPase activity was subtracted from the GTPase activity observed in the presence of fMLF to calculate the increase in GTPase activity caused by fMLF. From each basal GTPase activity value, the GTPase activity observed in the presence of CsH was subtracted to obtain the decrease in GTPase activity caused by CsH. Kinetic data were analyzed for best fit to single-site and two-site saturation curves. All data fit best to single-site saturation curves. To obtain GTP turnover numbers, GTPase activities (expressed as pmol/mg/min) were divided by the Bₘax total (pmol/mg) of ligand-regulated GTP₅S-binding of the respective membrane (see Table II) because this is a more precise measure of fusion protein expression level than receptor agonist binding (see Table I and Fig. 4). V₅₀max is the sum of fMLF- and CsH-regulated GTPase activity observed in the presence of fMLF. Efficacy was calculated based on the ratio of % binding of ligand-regulated GTP₅S-binding to the fusion protein to the % binding of ligand-regulated GTP₅S-binding to the respective membrane (see Table II).

**DISCUSSION**

Highly Efficient Receptor-G Protein Coupling in a FPR-G₁α Fusion Protein—The aim of our study was to quantitatively analyze the coupling of the FPR to the three G₁ isoforms. The fusion protein technique provides a defined 1:1 stoichiometry of the signaling partners and is a sensitive system for dissecting differences in the coupling of a given GPCR to different Gα isoforms (7, 43). However, because the fusion of a receptor C terminus to the Gα N terminus is artificial, one may be concerned that the fusion substantially alters the properties of the receptor and the G protein as well as their coupling to each other. Moreover, in certain expression systems, G₁ protein-coupled receptors cannot only couple to their fused G₁α partner but also to the endogenous G proteins of the host cells (50, 51).

To address these concerns, we compared the coupling of the FPR to G₁α in the fused and nonfused state and visualized activated G proteins by photoaffinity labeling. There were only minor differences in FPR-G₁αₐ coupling between the fused and nonfused state (Tables I and II), and there is no cross-talk between the fused FPR and G proteins of the host cell (Fig. 4). Thus, the fusion protein technique is a valid approach to analyze potential differences in the coupling of the FPR to G₁ isoforms.

In the co-expression system, there is an ~100-fold molar excess of G₁α over FPR as assessed by quantitative immunoblotting with defined standards (Figs. 4C and 5). Despite the large excess of G protein relative to receptor in the co-expression system and the high absolute expression levels of FPR and G₁α, the signaling efficiency of the co-expression system as assessed by the Bₘax of ligand-regulated GTP₅S-binding was not greater than that of the fusion protein system, which has only a 1:1 stoichiometry of receptor to G protein and a much lower G protein expression level (Table II). These data show...
that in the co-expression system, the vast majority of G proteins is not engaged in coupling, whereas in the fusion protein system, most if not all G proteins participate in coupling. Thus, the fusion induces an optimal positioning of Gs relative to the FPR. Similar conclusions were obtained for β2AR-Gs and Gs-α1isoforms, which differ from each other in GDP affinity, do not have a specific impact on the constitutive activity. Based on the above findings and considerations, we predicted that the FPR coupled to Gs-α1 would have a higher degree of constitutive activity than the FPR coupled to Gs-α3. Surprisingly, however, the relative inhibitory effects of CSH at the total ligand-regulated GTP-γ-S binding and GTP hydrolysis were very similar among the three Gs-α isoforms (Tables II–IV). Another indicator for increased constitutive activity of a GPCR is elevated efficacy and potency of partial agonists (7, 56, 57). However, we could not detect consistent increases in these parameters for FPR-Gs-α as compared with FPR-Gs-α1 and FPR-Gs-α2 (Table IV). Taken together, our data show that the different Gs-α isoforms, which differ from each other in GDP affinity, do not have a specific impact on the constitutive activity of the FPR. These data clearly demonstrate that the observations made for the coupling of Gs-α splice variants to the β2AR cannot be readily extrapolated to other GPCR-G protein pairs. Possibly, the intrinsic constitutive activity of the FPR is so high that the modulation of constitutive activity by GDP affinity of Gs-α isoforms is too subtle to become effective. The lack of substantial differences in the coupling of the FPR to Gs-α isoforms is somewhat unexpected in view of the fact that many GPCRs show differences in coupling efficiency to Gs isoforms, that Gs-α2 is the major G protein in phagocytes, and that Gs-α1 is not expressed in these cells (27, 29, 31–34). Of interest, the first, second, and third intracellular loops as well as the C terminus of the FPR are involved in G protein coupling (9, 60), whereas for most other receptors, the areas involved in G protein coupling appear to be more confined (61–63). Thus, the extensive contact area of the FPR with G proteins may provide the structural basis for the lack of differential coupling to Gs-α isoforms. Based on our findings, one can assume that in vivo, Gs-α1 and Gs-α3 are used interchangeably by the FPR. Stoichiometry of FPR-Gs Coupling: Implications for Signal-
bacteria, the concentration of formyl peptide increases, and more G proteins are activated, resulting in stimulation of cytotoxic cell functions such as lysosomal enzyme release and superoxide radical production and destruction of bacteria (8, 20, 21). Thus, linear signal transfer in phagocytes may help to prevent premature activation of cytotoxic cell functions and harmful destruction of host tissue.

Finally, the results of our present study indirectly provide information about the FPR expression level in vivo. Most GPCRs are expressed at levels in the fmol/mg range (see, for example, Refs. 40, 65, and 66), whereas the FPR is expressed at levels in the pmol/mg. Thus, the FPR is presumably a GPCR with one of the highest physiological expression levels.

In conclusion we have shown that the human FPR couples to the G proteins Gα1i, Gα2i, and Gα3i with similar efficiency. By taking advantage of the defined 1:1 stoichiometry of GPCR and Ga in fusion proteins, we obtained insights into quantitative aspects of FPR-G protein coupling that could not have been achieved by another approach. Our data suggest that the FPR activates Ga proteins in a linear fashion and not catalytically.

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