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ORIGINAL ARTICLE

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Functional differences between human formyl peptide receptor isoforms 26, 98, and G6

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Abstract The formyl peptide receptor (FPR) is expressed in neutrophils, couples to G_i-proteins and activates phospholipase C, chemotaxis and cytotoxic cell functions. FPR isoforms 26, 98, and G6 differ from each other in amino acids 101, 192 and 346 (FPR-26: V101, N192, E346; FPR-98: L101, N192, A346; FPR-G6: V101, K192, A346), but the functional significance of those structural differences is unknown. In order to address this question, we analyzed FPR-26, FPR-98 and FPR-G6 by co-expressing recombinant FLAG epitope-tagged FPRs with the G-protein $G_i\alpha_2\beta_1\gamma_2$ in Sf9 insect cells and measured high-affinity agonist binding and guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS) binding. The B_{max} values of high-affinity agonist binding with FPR-98 and FPR-G6 were much lower than with FPR-26. FPR-98 and FPR-G6 activated considerably fewer G_i-proteins, and were much less constitutively active, than FPR-26. Whereas FPR-26 migrated as a monomer in SDS polyacrylamide electrophoresis, FPR-98 and FPR-G6 migrated as dimers and tetramers. In terms of immunoreactivity, FRP-98 and FPR-G6 were expressed at higher levels than FPR-26. Single amino acid exchanges at positions 101 (V→L), 192 (N→K) and 346 (E→A) in FPR-26 revealed that E346 accounts for FPR-26 migrating as a monomer and the high constitutive activity of FPR-26. The V101L, N192K and E346A exchanges all reduced high-affinity agonist binding and the number of G_i-proteins activated by FPR-26. We conclude that (i) FPR isoforms 98 and G6 exhibit a partial G_i-protein coupling defect relative to FPR-26 and that (ii) E346 critically determines constitutive activity, G_i-protein coupling and physical state of FPR-26.

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Abbreviations *CsH* cyclosporin H · *FMLP N*-formyl-*L*-methionyl-*L*-leucyl-*L*-phenylalanine · *FPR* formyl peptide receptor · *GPCR* G-protein-coupled receptor · *GTPγS* guanosine 5'-*O*-(3-thiotriphosphate)

Introduction

Bacteria such as *Escherichia coli* and *Staphylococcus aureus* produce the formyl peptide FMLP that binds to specific FPRs expressed in the plasma membrane of neutrophils (Seifert and Schultz 1991; Murphy 1994; Prossnitz and Ye 1997; Rickert et al. 2000). Upon binding of FMLP, the FPR undergoes a conformational change from an inactive (R) state to an active (R*) state. In the R* state, the FPR promotes the GDP/GTP exchange at G_i-proteins, resulting in activation of phospholipase C-β and phosphatidylinositol-3-kinase. As outcome of these signaling events, neutrophils undergo chemotaxis towards the FMLP-producing bacteria, release reactive oxygen species and lysosomal enzymes and destroy the invading bacteria.

The human FPR exists in various isoforms, FPR-26, FPR-98 and FPR-G6, respectively (Boulay et al. 1990; Murphy et al. 1993). These FPR isoforms differ from each other in amino acid positions 101 (localized at the top of the third transmembrane domain), 192 (localized in the center of the second extracellular loop) and 346 (localized at the extreme C-terminus) (FPR-26: V101, N192, E346; FPR-98: L101, N192, A346; FPR-G6: V101, K192, A346) (Fig. 1). However, little is known about functional differences between FPR isoforms. FPR-26 reconstituted with the G-protein $G_i\alpha_2\beta_1\gamma_2$ in Sf9 insect cells possesses high constitutive activity, i.e., a high rate of agonist-independent isomerization from the R- to the R* state (Wenzel-Seifert et al. 1998). Experimentally, this high constitutive activity was unmasked by Na^+ and by the inverse agonist CsH. Specifically, Na^+ and CsH reduce the high basal

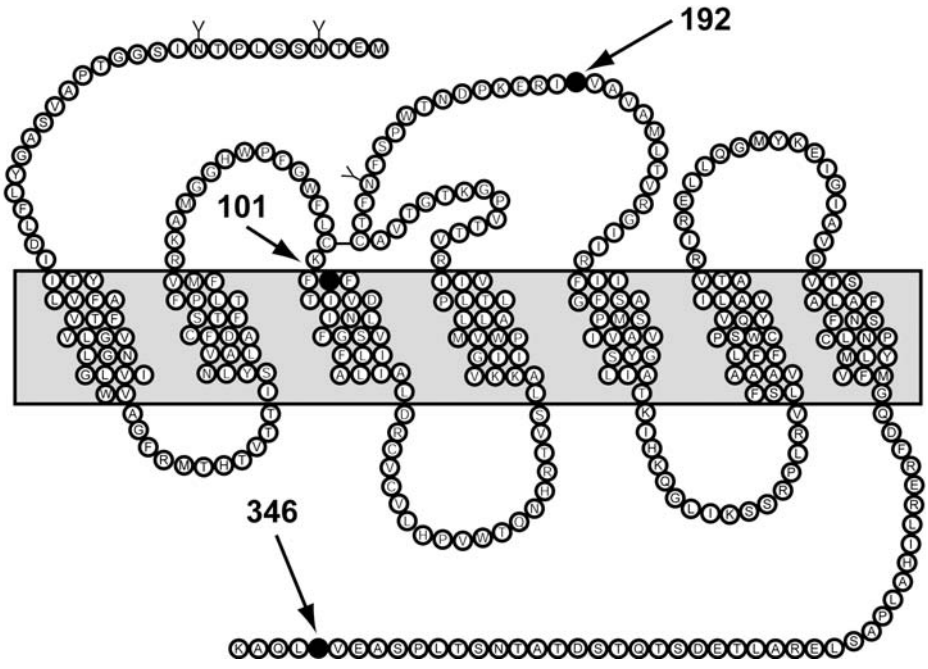

Fig. 1 Amino acid sequences of FPR isoforms. The two-dimensional structure of FPR-26 is shown. Amino acids are given in the one letter code. The FPR N-terminus (*top*) faces towards the extracellular space; the FPR C-terminus (*bottom*) faces towards the cytosol. The transmembrane domains are included in the *boxed area*. Extracellular consensus sites for *N*-glycosylation are shown with a *Y*. Amino acid positions 101, 192 and 346 are indicated with *filled black circles*. FPR-26: V101, N192, E346; FPR-98: L101, N192, A346; FPR-G6: V101, K192, A346. To analyze the effects of amino acid substitutions at positions 101, 192 and 346, the V101L exchange, N192 K exchange and E346A exchange were introduced into the FPR-26 sequence

GTPγS binding to G_i-proteins by stabilizing the R state of FPR-26. The large inhibitory effect of CsH on G_i-protein activation in Sf9 cell membranes expressing FPR-26 contrasts to the lack of effect of CsH on G_i-protein activation in membranes from differentiated HL-60 leukemia cells that express the FPR endogenously (Wenzel-Seifert and Seifert 1993; Wenzel-Seifert et al. 1998). An explanation for this discrepancy could be that various FPR isoforms differ from each other in constitutive activity and that HL-60 membranes express exclusively or predominantly FPR isoforms with low constitutive activity. Our study aim was to uncover biochemical differences between FPR-26, FPR-98 and FPR-G6. In addition, we analyzed the role of individual amino acids in FPR function by introducing single amino acid exchanges (V101L, N192 K and E346A, respectively) into the FPR-26 sequence.

Materials and methods

The cDNAs of FPR-26 and FPR-98 in pCDM 8 were kindly provided by Dr. F. Boulay (Laboratoire de Biochimie, CNRS, Grenoble, France). Sources of other materials were described before (Wenzel-Seifert et al. 1998, 1999). The cDNAs of FPR-G6, FPR-V101L, FPR-N192 K and FPR-E346 were constructed by site-directed mutagenesis according to published procedures (Wenzel-Seifert et al. 1998, 1999). Culture of Sf9 cells and membrane preparation were performed as described (Seifert et al. 1998; Wenzel-Seifert et al. 1998, 1999). [^{3}H]FMLP saturation binding was determined as described (Wenzel-Seifert et al. 1998, 1999). [^{35}S]GTPγS binding experiments were conducted as described (Wenzel-Seifert et al. 1998, 1999). SDS polyacrylamide electrophoresis and immunoblotting were performed as described (Wenzel-Seifert et al. 1998, 1999). Membranes were dissolved in sample buffer at room temperature and were not heated to prevent formation of artificial dimers. Protein concentrations were determined using the Bio-Rad DC protein assay kit (Bio-Rad; Hercules, CA, USA). Data were analyzed by non-linear regression, using the Prism 3.02 program (Graphpad-Prism; San Diego, CA, USA).

Results

Analysis of the expression of FPR isoforms, FPR-V101L, FPR-N192 K, FPR-E346A and $G_i\alpha_2$ by immunoblotting

The expression of FLAG epitope-tagged FPRs in Sf9 membranes was examined in immunoblots with the M1 monoclonal antibody. As reported before (Wenzel-Seifert et al. 1998; Seifert and Wenzel-Seifert 2001b), FPR-26 migrated as a diffuse glycosylated band with an apparent molecular mass of ~40 kDa in SDS polyacrylamide gels (Fig. 2). There was no evidence for dimer formation with FPR-26. In marked contrast, FPR-98 and FPR-G6 migrated as a diffuse glycosylated band with a molecular mass of ~60–100 kDa and, to a lesser extent, as a ~150 kDa band. These data indicate that FPR-98 and FPR-G6 do not exist as monomers but rather form SDS-resistant dimers (and tetramers). Similar to FPR-26, FPR-V101L and FPR-N192 K migrated as monomers, whereas FPR-E346A, like FPR-98 and FPR-G6, migrated as dimer (and tetramer). Based on the intensity of immunoreactive bands, FPR-98, FPR-G6 and FPR-E346A were expressed at several-fold higher levels than FPR-26, FPR-V101L and FPR-N192 K. In agreement with previous studies on various GPCRs (Wenzel-Seifert et al. 1998, 1999; Wenzel-Seifert and Seifert 2000; Seifert and Wenzel-Seifert 2001a, 2001b), the expression of $G_i\alpha_2$ was similar in Sf9 membranes expressing FPR-26, FPR-98, FPR-G6, FPR-V110L, FPR-N192 K and FPR-E346A (data not shown).

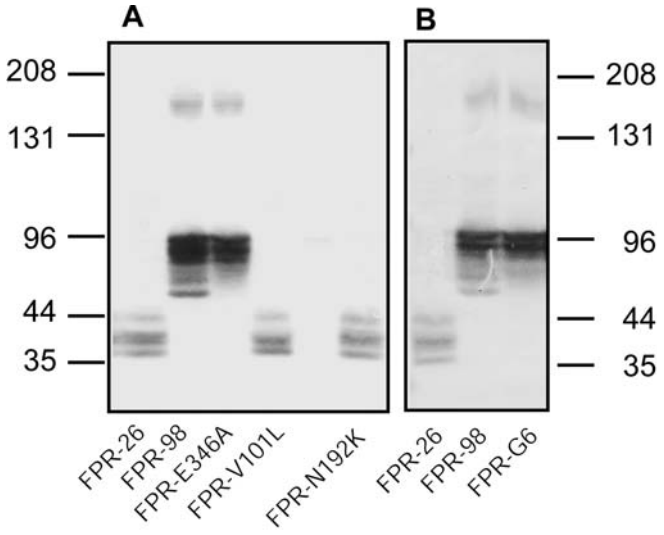

Fig. 2A, B Analysis of the expression of FPR constructs in Sf9 cell membranes by immunoblotting. Sf9 membranes expressing various FPR constructs plus $G_i\alpha_2\beta_1\gamma_2$ were prepared. Fifty micrograms of protein were loaded onto each lane. Membrane proteins were separated by SDS polyacrylamide electrophoresis and probed with the M1 monoclonal antibody (anti-FLAG Ig) as described in Materials and methods. *Numbers at the left margin* of panel **A** and at the *right margin* of panel **B** indicate molecular masses of marker proteins. Shown are the horseradish peroxidase-reacted Immobilon P membranes of gels containing 10% (w/v) acrylamide. **A** and **B** show immunoblots performed with different membrane preparations

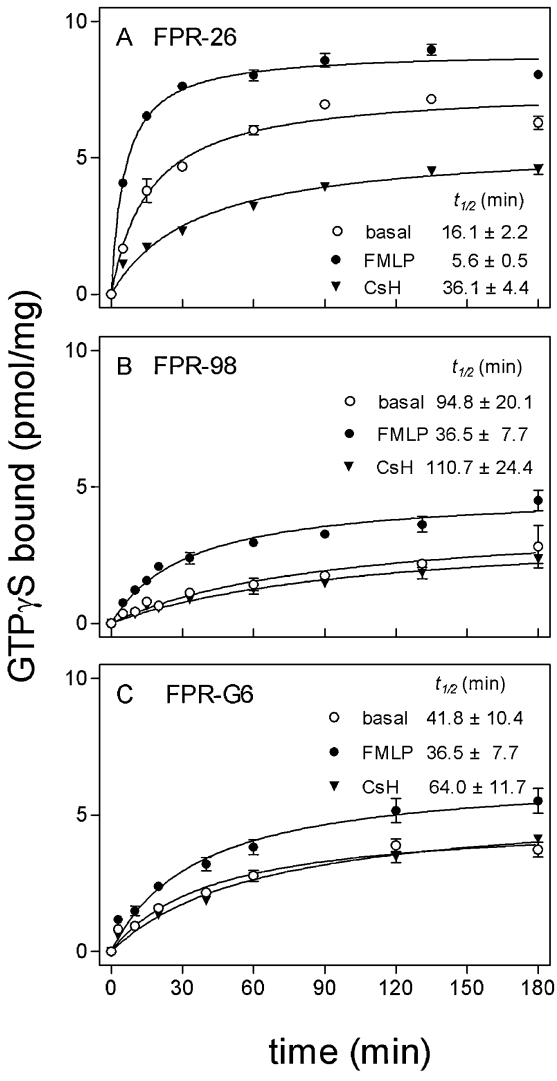

Fig. 3A–C Time course of GTPγS binding in Sf9 cell membranes expressing FPR-26, FPR-98 or FPR-G6 plus $G_i\alpha_2\beta_1\gamma_2$. Membranes expressing various FPR isoforms plus $G_i\alpha_2\beta_1\gamma_2$ were prepared. [^{35}S]GTPγS binding experiments in membranes expressing **A** FPR-26, **B** FPR-98 and **C** FPR-G6 plus $G_i\alpha_2\beta_1\gamma_2$ were carried out as described in Materials and methods. Membranes were incubated for the periods of time indicated on the abscissa in the presence of solvent (basal) (*white circles*), 10 µM FMLP (*black circles*) or 10 µM CsH (*black triangles*). The total GTPγS concentration was 10 nM (1 nM [^{35}S]GTPγS plus 9 nM unlabeled GTPγS). Reaction mixtures also contained 1 µM GDP. Data shown are the means ± SD of three experiments performed in triplicates. Binding data were analyzed by non-linear regression and were best fitted (F test) to monophasic saturation curves

Analysis of FPR isoforms, FPR-V101L, FPR-N192 K and FPR-E346A by [^{3}H]FMLP saturation binding

Next, we studied high-affinity agonist binding. This assay provides a measure for functionally active FPRs coupled to G_i-proteins (Wenzel-Seifert et al. 1998, 1999). Sf9 membranes expressing FPR-26 bound the agonist [^{3}H]FMLP with a K_d value of 3.3±0.5 nM and a B_{max} of 0.53±0.11 pmol/mg (means ± SD, n=4). In membranes expressing FPR-98, FPR-G6, FPR-V101L, FPR-N192 K and FPR-E346A, the K_d values for [^{3}H]FMLP ranged between 0.8 and 1.4 nM, indicating that those FPRs can exist in a state of high agonist-affinity. However, the B_{max} values were reduced to 0.03±0.01 pmol/mg (FPR-98), 0.02±0.01 pmol/mg (FPR-G6), 0.07±0.02 pmol/mg (FPR-V101L), 0.06±0.02 pmol/mg (FPR-N192 K) and 0.05±0.01 pmol/mg (FPR-E346A) (means ± SD, n=3).

Analysis of FPR isoforms, FPR-V101L, FPR-N192 K and FPR-E346A by [^{35}S]GTPγS binding

The FPR catalyzes GDP/GTP exchange at G_i-proteins which process is monitored by binding of the GTP analog, [^{35}S]GTPγS (Gierschik et al. 1991; Wenzel-Seifert et al. 1998, 1999). In membranes expressing FPR-26, the agonist FMLP accelerated the GTPγS association rate by ~3-fold, whereas the inverse agonist CsH reduced the GTPγS association rate by more than 2-fold (Fig. 3A). Compared to membranes expressing FPR-26, the GTPγS association kinetics in membranes expressing FPR-98 and FPR-G6 were much slower under all conditions (Fig. 3B, C). In addition, the inverse agonist CsH had only minimal inhibitory effect on GTPγS binding in membranes expressing FPR-98 and FPR-G6. Moreover, the absolute GTPγS binding values with FPR-98 and FPR-G6 were lower than with FPR-26.

To corroborate the differences in the functional activities of FPR isoforms, we conducted GTPγS saturation bind-

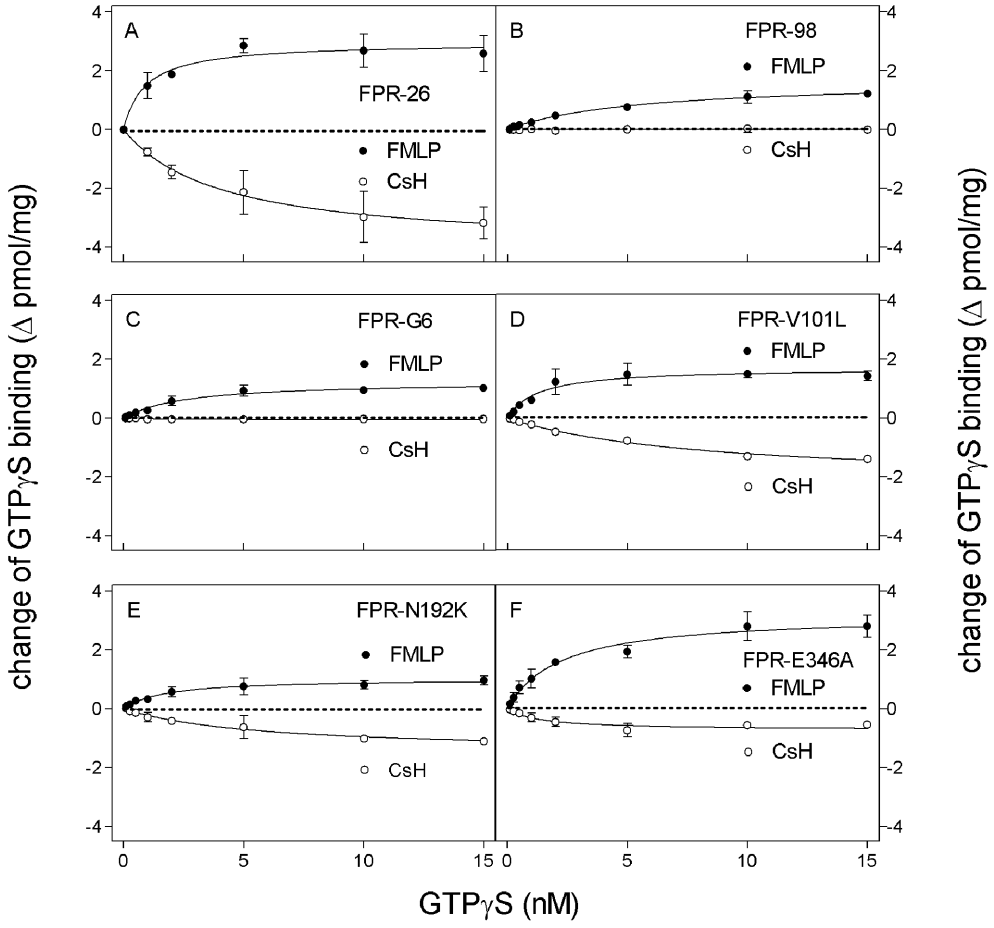

Fig. 4A–F GTPγS saturation binding studies in Sf9 cell membranes various FPR constructs plus $G_i\alpha_2\beta_1\gamma_2$. Membranes expressing various FPR constructs plus $G_i\alpha_2\beta_1\gamma_2$ were prepared. [^{35}S]GTPγS binding experiments in membranes expressing **A** FPR-26, **B** FPR-98, **C** FPR-G6, **D** FPR-V101L, **E** FPR-N192 K or **F** FPR-E346A plus $G_i\alpha_2\beta_1\gamma_2$ were carried out as described in Materials and methods. Reaction mixtures contained 1 µM GDP, 0.5–2 nM [^{35}S]GTPγS plus unlabeled GTPγS to achieve final GTPγS concentrations of 0.5–15 nM as indicated on the abscissa and solvent (basal), 10 µM FMLP or 10 µM CsH. For each GTPγS concentration, basal GTPγS binding was subtracted from GTPγS binding in the presence of FMLP to obtain FMLP-stimulated GTPγS binding (*black circles*). GTPγS binding in the presence of CsH was subtracted from basal GTPγS binding to obtain CsH-inhibited GTPγS binding (*white circles*). The *dashed lines* represent extrapolations of basal GTPγS binding. The ligand-regulated GTPγS binding is the difference between minimum CsH-inhibited GTPγS binding and maximum FMLP-stimulated GTPγS binding. Data shown are the means ± SD of three experiments performed in triplicates. Binding data were analyzed by non-linear regression and were best fitted (F test) to monophasic saturation curves

ing studies. The B_{max} value of ligand-regulated GTPγS binding, i.e. the difference between minimum CsH-inhibited and maximum FMLP-stimulated GTPγS binding (Wenzel-Seifert et al. 1998, 1999) was ~4–5-fold higher for FPR-26 (B_{max}, 7.0±0.6 pmol/mg) than for FPR-98 (B_{max}, 1.7±0.3 pmol/mg) and FPR-G6 (B_{max}, 1.3±0.2 pmol/mg) (Fig. 4A–C).

To further analyze the constitutive activity of FPR isoforms, we studied the effect of Na^+ on GTPγS binding. At all FPR isoforms, Na^+ reduced the constitutive GTPγS binding in a concentration-dependent manner (Fig. 5). The IC_{50} values for the effect of Na^+ on basal GTPγS binding were similar for the FPR isoforms studied (FPR-26, 27 mM; 95% confidence interval, 16–71 mM) (FPR-98, 52 mM; 95% confidence interval, 31–146 mM) (FPR-G6, 40 mM; 95% confidence interval, 15–146 mM), indicating that the Na^+-affinity of the three FPR isoforms is similar. However, the inhibitory effect of Na^+ on constitutive GTPγS binding was much larger in membranes expressing FPR-26 than in membranes expressing FPR-98 and FPR-G6.

To elucidate the molecular basis for the functional differences between FPR-26, FPR-98 and FPR-G6, we analyzed GTPγS saturation binding in membranes expressing FPR-V101L, FPR-N192 K and FPR-E346A. With respect to the maximum number of G_i-proteins activated, FPR-26 (B_{max} of ligand-regulated GTPγS binding, 7.0±0.6 pmol/mg) surpassed FPR-V101L (B_{max} 3.9±0.3 pmol/mg), FPR-N192 K (B_{max} 2.5±0.2 pmol/mg) and FPR-E346A (B_{max} 3.9±0.5 pmol/mg). The relative inhibitory effects of CsH on the ligand-regulated GTPγS binding in membranes expressing FPR-V101L and FPR-N192 K amounted to 56.7% and 59.6%, respectively, and were similar to the inhibitory effect of CsH in membranes expressing FPR-26 (57.9% of ligand-regulated GTPγS binding) (compare Fig. 4A, D and E). Compared to membranes expressing FPR-26, FPR-V101L and FPR-N192 K, the inhibitory effect of CsH in membranes expressing FPR-E346A was strongly re-

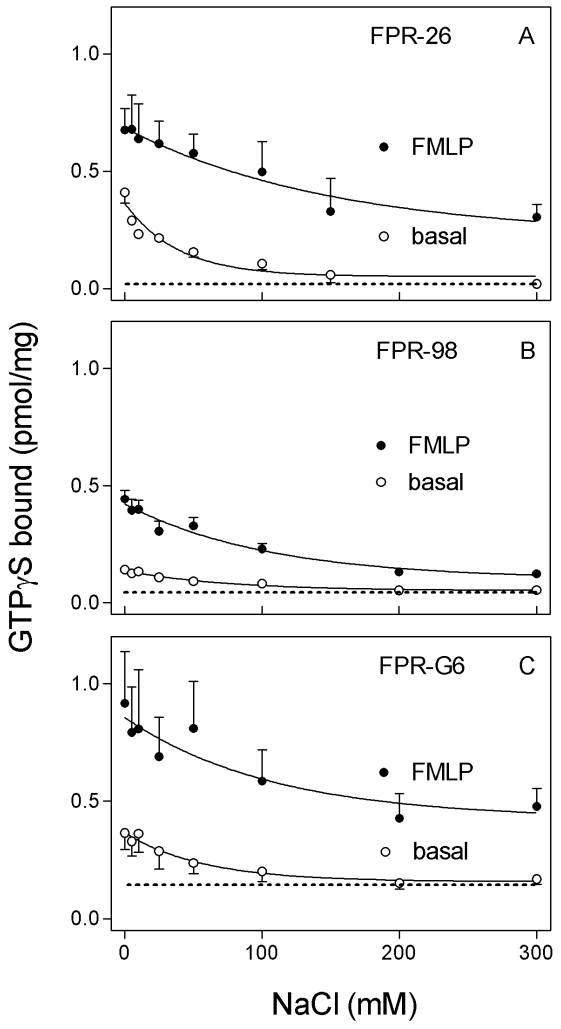

Fig. 5A–C Effects of NaCl on basal and FMLP-stimulated GTPγS binding Sf9 cell membranes expressing FPR-26, FPR-98 or FPR-G6 plus $G_i\alpha_2\beta_1\gamma_2$. Membranes expressing various FPR constructs plus $G_i\alpha_2\beta_1\gamma_2$ were prepared. [^{35}S]GTPγS binding experiments in membranes expressing **A** FPR-26, **B** FPR-98 or **C** FPR-G6 plus $G_i\alpha_2\beta_1\gamma_2$ were carried out as described in Materials and methods. Reaction mixtures contained 1 µM GDP, 0.4 nM [^{35}S]GTPγS (FPR-26) or 2 nM [^{35}S]GTPγS (FPR-98 and FPR-G6) and solvent (basal) or 10 µM FMLP. In addition, reaction mixtures contained NaCl at the concentrations indicated on the abscissa. Data shown are the means ± SD of three experiments performed in triplicates. Solvent (basal) (*white circles*); FMLP (10 µM) (*black circles*). The *dashed lines* represent extrapolations of the basal GTPγS binding observed in the presence of 300 mM NaCl. Data were analyzed by non-linear regression and were best fitted to monophasic exponential decay functions (F test)

duced (18.7% of ligand-regulated GTPγS binding) (compare Fig. 4A, D, E and F).

Discussion

Our present study shows that FPR-26 is considerably more efficient than FPR-98 and FPR-G6 at coupling to G_i-proteins in terms of high-affinity agonist binding and GTPγS binding (Figs. 3, 4). Moreover, FPR-26 possesses a much higher constitutive activity than FPR-98 and FPR-G6 as is shown by the larger inhibitory effects of Na^+ and the inverse agonist CsH on basal GTPγS binding in membranes expressing FPR-26 than in membranes expressing FPR-98 and FPR-G6 (Figs. 3, 4, 5). These data contrast to the fact that in terms of immunoreactivity, FPR-98 and FPR-G6 are expressed at substantially higher levels than FPR-26 (Fig. 2). Two explanations could account for the discrepancies between functional activity of FPR isoforms and expression level. First, it is possible that the majority of the FPR-98- and FPR-G6 molecules exist in a state of very low FMLP-affinity that was not detected in our binding assay and is uncoupled from G-proteins (Gierschik et al. 1989; Quehenberger et al. 1992). Second, it is possible that FPR-98 and FPR-G6 exhibit a folding defect, resulting in the production of large quantities of functionally inactive FPR aggregates and a partial G_i-protein coupling defect. Thus, the FPR-26 monomers may reflect functionally active GPCRs, whereas the FPR-98- and FPR-G6 dimers and tetramers may represent misfolded proteins (Fig. 2). In accordance with the latter interpretation, the FPR-C126 W mutant associated with juvenile periodontitis and exhibiting a complete G_i-protein coupling defect, shows multiple high molecular mass species in SDS polyacrylamide electrophoresis as well (Seifert and Wenzel-Seifert 2001b).

It is unlikely that the partial G_i-protein coupling defect of FPR-98 and FPR-G6 is an artifact of the insect cell expression system for several reasons. First, there is no evidence for alteration of the biochemical properties of various chemoattractant receptors in Sf9 cells compared to neutrophils or HL-60 leukemia cells (Klinker et al. 1996; Wenzel-Seifert et al. 1998, 1999; Seifert and Wenzel-Seifert 2001a). Second, the G_i-protein coupling defect of FPR-C126 W in Sf9 cells is reproduced by a very similar mutant, FPR-C126S, in a mammalian expression system (CHO cells) (Miettinen et al. 1999; Seifert and Wenzel-Seifert 2001b). Third, functionally active FPR-26 is expressed in HEK-293 cells (Wenzel-Seifert et al. 1998), but we failed to express functionally active FPR-98 and FPR-G6 in various strains of HEK-293 cells, using different transfection protocols (K. Wenzel-Seifert and R. Seifert, unpublished results). Presumably, the higher GPCR expression levels obtained in Sf9 cells relative to HEK-293 cells enabled us to detect some G_i-protein coupling of FPR-98 and FPR-G6, rendering the insect cells a more suitable expression system for these FPR isoforms than mammalian cells.

The greater functional activity of FPR-26 relative to FPR-98 and FPR-G6 can be attributed to the combined presence of V101, N192 and E346. The exchange of these amino acids against L101, K192 and A346, respectively, reduces the functional activity of the FPR constructs to different extents in terms of high-affinity agonist binding and GTPγS binding (Fig. 4). The combined exchange of two of these amino acids (V101L and E346A in FPR-98; N192 K and E346A in FPR-G6) reduces the functional activity of the GPCRs even further (Figs. 3, 4, 5). These data

indicate that the amino acids at positions 101, 192 and 346 all contribute to FPR function. Since the C-terminus of the FPR is directly involved in G_i-protein coupling (Bommakanti et al. 1993), it is possible that an amino acid exchange in the extreme C-terminus (position 346) alters the efficiency of the FPR at interacting with G_i-proteins. However, the amino acids at positions 101 (top of the third transmembrane domain) and 192 (center of the second extracellular loop) (Fig. 1) cannot directly participate in G_i-protein coupling (Bommakanti et al. 1995). Thus, one has to postulate propagation of local conformational changes over relatively long distances to the G_i-protein-coupling intracellular domains (Bommakanti et al. 1993, 1995).

So far, little is known about the role of specific amino acids in the C-terminus of GPCRs for their oligomerization. Our present data clearly show that an E→A exchange at position 346 in the extreme C-termimus of the FPR critically determines oligomerization of the FPR (Figs. 1 and 2). It is conceivable that A346 facilitates hydrophobic interactions between the C-termini of two FPR molecules and, thereby, triggers dimerization. This notion is supported by the fact that A346 is surrounded by two other hydrophobic amino acids, namely V345 and L347 (Fig. 1). An alternative explanation for the high molecular mass species in membranes expressing FPR-98, FPR-G6 and FPR-E346A could be complexes of FPRs with insect proteins, e.g., insect G-proteins. However, this explanation is unlikely since Sf9 cells do not express mammalian-type G_i-proteins, and FPRs do not couple to insect cell G-proteins (Quehenberger et al. 1992; Wenzel-Seifert et al. 1998, 1999).

Previous studies have shown that changes in the structure of GPCR C-terminus, including single amino acid exchanges, alter the constitutive activity of GPCRs (Prezeau et al. 1996; Jin et al. 1997; Kopin et al. 2000). Our data show that the E→A exchange at position 346 in the extreme C-terminus of the FPR largely reduces the constitutive activity of FPR isoforms (Fig. 4). There may be a functional link between FPR dimerization and constitutive activity. Specifically, one could envisage that FPR dimerization constrains the mobility of FPR molecules in such a way that the R/R* isomerization is impaired. Alternatively or additionally, dimerization could reflect a folding defect of FPR. It is conceivable that the functional outcome of such a folding defect is impaired R/R* isomerization as well. To our knowledge, this is the first indication for an inverse relation between GPCR dimerization and constitutive activity of GPCRs.

FPR isoforms with severely impaired G_i-protein coupling compared to FPR-26 are associated with localized juvenile periodontitis which is caused by *Actinobacillus actinomycetemcomitans* (Gwinn et al. 1999; Seifert and Wenzel-Seifert 2001b). These findings raise the intriguing question whether FPR isoforms with a partial G_i-protein coupling defect relative FPR-26, i.e., FPR-98 and FPR-G6, are linked to acute and/or chronic diseases associated with bacterial infections. Thus, future studies will have to determine the allele frequencies of FPR-26, FPR-98 and FPR-G6 in healthy humans and in diseases such as mucoviscidosis, chronic obstructive lung disease, ulcerative colitis, endocarditis and sepsis. The slow kinetics of G_i-protein activation by FPR-98 and FPR-G6 also raise the question whether these GPCRs play specific roles in mediating sustained responses of neutrophils to FMLP. Different functional activities of FPR isoforms together with differential expression of FPR isoforms in various individuals could provide the molecular basis for the fact that the FMLP-responsiveness of neutrophils from different individuals varies vastly (Seifert et al. 1991). Moreover, our present data could provide an explanation for the previously observed lack of inhibitory effect of the inverse agonist CsH on G_i-protein activation in HL-60 cell membranes (Wenzel-Seifert and Seifert 1993). Particularly, HL-60 cells may express FPR-98 and/or FPR-G6 at much higher levels than the highly constitutively active FPR-26. Finally, we will learn more about the physiological functions of FPR isoforms by studying transgenic mice overexpressing defined FPR isoforms and FPR knock-out mice (Gao et al. 1999) in which human FPR isoforms are expressed by retroviral infection.

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