Identification of Transmembrane Domain Residues Determinant in the Structure-Function Relationship of the Human Platelet-activating Factor Receptor by Site-directed Mutagenesis*

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Platelet-activating factor (PAF) is a potent phospholipid mediator that produces a wide range of biological responses. The PAF receptor is a member of the seven-transmembrane GTP-binding regulatory protein-coupled receptor superfamily. This receptor binds PAF with high affinity and couples to multiple signaling pathways, leading to physiological responses that can be inhibited by various structurally distinct PAF antagonists. We have used site-directed mutagenesis and functional expression studies to examine the role of the Phe\(^97\) and Phe\(^98\) residues located in the third transmembrane helix and Asn\(^{285}\) and Asp\(^{289}\) of the seventh transmembrane helix in ligand binding and activation of the human PAF receptor in transiently transfected COS-7 cells. The double mutant FFGG (Phe\(^97\) and Phe\(^98\) mutated into Gly residues) showed a 3-4-fold decrease in affinity for PAF, but not for the specific antagonist WEB2086, when compared with the wild-type (WT) receptor. The FFGG mutant receptor, however, displayed normal agonist activation, suggesting that these two adjacent Phe residues maintain the native PAF receptor conformation rather than interacting with the ligand. On the other hand, substitution of Ala for Asp\(^{289}\) increased the receptor affinity for PAF but abolished PAF-dependent inositol phosphate accumulation; it did not affect WEB2086 binding. Substitution of Asn for Asp\(^{289}\), however, resulted in a mutant receptor with normal binding and activation characteristics. When Asn\(^{289}\) was mutated to Ala, the resulting receptor was indistinguishable from the WT receptor. Surprisingly, substitution of Ile for Asn\(^{285}\) led to a loss of ligand binding despite normal cell surface expression levels of this mutant, as verified by flow cytometric analysis. Our data suggest that residues 285 and 289 are determinant in the structure and activation of the PAF receptor but not in direct ligand binding, as had been recently proposed in a PAF receptor molecular model.

Platelet-activating factor (PAF) is a potent phospholipid mediator that is involved in a variety of biological activities related to inflammatory and immune responses (1) as well as cardiovascular, reproductive, respiratory, and nervous system physiology (2). The PAF structure has been identified as 1-O-alkyl-2-O-acetyl-sn-glycero-3-phosphocholine (3, 4). It is released by stimulated basophils, platelets, macrophages, polymorphonuclear neutrophils, and other cell types (1, 2). The PAF structural requirement is highly specific for its biological actions, which are mediated through binding and activation of a specific, high-affinity receptor on the target cell surface. PAF binding has been found on several cell types, and cDNA cloning from various sources revealed that the PAF receptor belongs to the G protein-coupled receptor superfamily (5–9). The PAF receptor couples with various second messenger systems, including phospholipase A\(_2\), C, and D activation (10–12) and the mitogen-activated protein kinase cascade (11, 13–15). PAF-dependent cellular responses can be inhibited by a variety of structurally distinct PAF antagonists (16).

The recent cloning of the PAF receptor has made possible the study of the structure-function relationship of this receptor, but little information has been published as yet. The cytoplasmic tail of the guinea pig PAF receptor has been shown not to be required for the forward signal transduction to multiple pathways but to play an essential role in the agonist-induced desensitization (17). Moreover, the human PAF receptor contains a single N-linked consensus glycosylation sequence in the N-terminal region of the third intracellular loop (7), in contrast to the guinea pig (18) and the rat (19) receptors, which have an additional NH\(_2\)-terminal consensus sequence for N-glycosylation. In this context, Streptococcus pneumoniae was recently shown to use the human PAF receptor for adherence to and invasion of host cells (20). It was later shown that this N-glycosylation site enhances bacterial binding to the PAF receptor but is not required for the interaction (21). Glycosylation at this site is necessary for efficient membrane trafficking of the PAF receptor but seems to have no role in receptor affinity and activation (21). In additional structural studies, we have demonstrated that mutation of two adjacent residues, Ala\(^{230}\) and Leu\(^{251}\), in the COOH-terminal region of the third intracellular loop led respectively to inactive and constitutively active phenotypes of the PAF receptor (22). Moreover, we showed that the highly conserved Asp\(^{253}\) residue in the second transmembrane helix is not involved in ligand binding but is necessary for G protein coupling of the human PAF receptor (23).

Recently, a molecular model of the PAF receptor was proposed based on the bacteriorhodopsin three-dimensional structure (24). In this model, the side chains of Asp\(^{253}\), Asn\(^{285}\), and...
Asp289 were adjacent to each other and oriented toward the central core of the receptor. These three residues were thought to form a negatively charged site that would attract the positively charged choline moiety of the PAF molecule by electrostatic forces. Conserved Asp residues in the third TMH, which were shown to participate in hydrophobic ligand interactions for other G protein-coupled receptors (25), are absent in the corresponding domain of the PAF receptor. Instead, two hydrophobic residues, Phe97 and Phe98, are similarly located, which could possibly interact with the acetyl group or the phospholipid chain of the PAF molecule. No experimental data are yet available on residues that might be part of the binding pocket of the PAF receptor. To address this question, we mutated the Phe97, Phe98, Asn285, and Asp289 residues of the PAF receptor, and properties of these mutants were compared with those of the wild-type (WT) PAF receptor in transiently transfected COS-7 cells. In this report, we suggest that the Phe97 and Phe98 residues may be involved in maintaining the native conformation of the PAF receptor and, moreover, that the PAF molecule does not bind to the Asn285 and Asp289 residues, contrary to what had been proposed (24). However, the two latter amino acids could be determinant in receptor conformation and activation.

EXPERIMENTAL PROCEDURES

Construction of the Mutant Receptor cDNAs and Expression Vectors—The PAF receptor cDNA derived from Kp132 (a generous gift from Dr. Richard Ye, The Scripps Research Institute, La Jolla, CA) (8) was subcloned into the pBR-cytomegalovirus expression vector (Invitrogen). Mutated receptors were constructed by polymerase chain reaction (26) using Kp132 as a template. To create the FFGG double mutant, we made the oligonucleotide 5′-GGGCTGCGCTGTTGCGCCGATCAACACCTAC-3′ and its reverse complement, which changes TTC(Phe) to CACCTAC-3′, and its reverse complement. To determine residues that might contribute to ligand binding and receptor activation of the human PAF receptor, we performed site-directed mutagenesis of the Phe97, Phe98, Asn285, and Asp289 residues and evaluated the properties of the mutant receptors in comparison with the WT PAF receptor in transiently transfected COS-7 cells. The reactions were terminated with the addition of perchloric acid followed by a 30-min incubation on ice. Inositol phosphates were extracted (28) and separated on Dowex AG1-X8 (Bio/Rad) columns (29). Total labeled inositol phosphates were then counted by liquid scintillation.

Flow Cytometric Studies—The N285I and WT receptors were subcloned in frame with the e-myc epitope in the pJ3M vector, kindly provided by Dr. J. Chernoff (Fox Chase Cancer Center, Philadelphia, PA) (30). The pJ3M-e-myc N285I and pJ3M-e-myc WT receptor constructs were transfected in COS-7 cells, which were harvested 48 h after transfection and subjected to flow cytometric analysis. Cells (2.5×10⁵) were washed twice in PBS and labeled with or without anti-e-myc antibody (9E10 hybridoma; American Type Culture Collection) at room temperature for 30 min. Cells were then washed with PBS and incubated at room temperature for an additional 30 min with fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Bio/Can). All measurements were performed on a FACScan flow cytometer (Becton-Dickinson).

RESULTS

To determine residues that might contribute to ligand binding and receptor activation of the human PAF receptor, we performed site-directed mutagenesis of the Phe97, Phe98, Asn285, and Asp289 residues and evaluated the properties of the mutant receptors in comparison with the WT PAF receptor in transiently transfected COS-7 cells. Fig. 1 shows a representation of the putative seven membrane-spanning domain topography of the PAF receptor and indicates the amino acids that were replaced in the mutant receptors.

Characterization of the Binding Properties of the Mutant Receptors—Binding characteristics of the WT and mutant receptors are summarized in Table I. Fig. 2 shows that the affinity of the specific PAF receptor antagonist WEB2086 for the indicated mutant receptors was unaltered compared with the WT receptor in competition binding experiments. Calculated receptor densities (Bmax), indicated as receptors per cell, were also similar for the different receptors, except for the N285I mutant (Table I). As is well known, PAF being a phospholipid, binding experiments using [³H]PAF constitute a large nonspecific component that renders reproducibility and interpretation of the results difficult (27). Since the affinity of the antagonist was the same for all the receptors, we used competition of [³H]WEB2086 by cold PAF to assess the PAF affinity for the different receptors and obtained high reproducibility between experiments. Fig. 3A shows the competition binding isotherms of [³H]WEB2086 by PAF for the indicated receptors. The WT receptor displayed a Kᵢ of 33 ± 4.6 nM for PAF, whereas the N285A and D289N mutants had corresponding values of 31 ± 4.1 and 34 ± 3.1 nM, respectively. Surprisingly, the N285I mutant did not display any specific WEB2086 or PAF binding. No significant specific binding above the control level (vector-
transfected cells) could be detected for this mutant in every single experiment (n = 4). A 3-fold higher affinity for PAF than the WT receptor was displayed by the D289A mutant receptor, which had a $K_i$ of 12.6 ± 2.3 nM. In contrast, the FFGG mutant receptor had almost a three-fold lower affinity for PAF, with a $K_i$ of 87 ± 5.7 nM. As these results indirectly assess PAF binding by displacing the [3H]WEB2086 probe, we then performed direct studies of PAF binding to further support our conclusions. Fig. 3 illustrates competition binding isotherms of PAF to the different receptors. As summarized in Table I, conclusions that can be drawn by the direct PAF binding studies are essentially the same as those from the displacement of [3H]WEB2086. The WT, N285A, and D289N receptors displayed the same affinity for PAF, with respective $K_i$ values of 12 ± 2.7, 9.8 ± 2.4, and 12.7 ± 3.1 nM. A 4-fold higher affinity for PAF than the WT receptor was displayed by the D289A mutant, whereas the FFGG double mutant showed approximately a 4-fold lower affinity for PAF than the WT receptor (Table I).

**Inositol Phosphate Accumulation in Mutant Receptor-transfected Cells**—The ability of the mutant receptors to transduce a signal was then tested by measuring IP accumulation following stimulation with graded concentrations of PAF. Fig. 4 illustrates the concentration-response curves of IP accumulation for the WT and mutant receptors in response to PAF concentrations from 0 to 10^{-6} M. PAF concentrations higher than 10^{-6} M were not used, as PAF has been shown to have nonreceptor-mediated effects at these concentrations (31), making the interpretation of results difficult. No significant difference could be detected between the activation of the FFGG, N285A, D289N, and WT receptors, in which IP production reached a plateau at $10^{-7}$ M of agonist with similar half-maximal effective concentration values (Table I). No IPs were produced by the N285I and D289A mutants over the entire range of PAF concentrations.

**Cell Surface Expression of the N285I Mutant Receptor**—To

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**Table I**

| Receptor       | $B_{max}$ WEB2086 | $K_i$ WEB2086 | $[3H]$WEB2086 + PAF $K_i$ | $[3H]$PAF + PAF $K_i$ | EC50 |
|----------------|-------------------|---------------|---------------------------|------------------------|------|
| Wild-type      | 802 ± 500         | 30 ± 2.1      | 33 ± 4.6                  | 12 ± 2.7               | 8.0 ± 1.2 |
| FFGG mutant    | 712 ± 640         | 39 ± 2.7      | 87 ± 5.7                  | 49 ± 6.9               | 7.8 ± 0.9 |
| N285A mutant   | 691 ± 725         | 21 ± 1.9      | 31 ± 4.1                  | 9.8 ± 2.4              | 11 ± 2.1 |
| N285I mutant   | ND                | ND            | ND                        | ND                     | ND   |
| D289A mutant   | 762 ± 484         | 23 ± 2.0      | 12 ± 2.3                  | 2.9 ± 0.9              | 6.5 ± 0.7 |
| D289N mutant   | 911 ± 071         | 34 ± 3.1      | 34 ± 3.1                  | 12.7 ± 3.1             | 12.7 ± 3.1 |

*ND, not detectable.*

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**Fig. 2.** Competition binding isotherms of [3H]-WEB2086 by WEB2086 in COS-7 cells. [3H]-WEB2086 binding was measured as indicated under “Experimental Procedures” on COS-7 cells transiently expressing the WT and the indicated mutant receptors. The results are representative of three independent experiments, the mean ± S.E. values of which are reported in Table I.

**Fig. 3.** Competition binding isotherms of [3H]-WEB2086 by PAF (A) and [3H]-PAF by PAF (B) in COS-7 cells. [3H]-WEB2086 and [3H]-PAF binding were determined as indicated under “Experimental Procedures” on COS-7 cells transiently expressing the WT and the indicated mutant receptors. The results are representative of three independent experiments, the mean ± S.E. values of which are reported in Table I.
verify whether the phenotype observed for the N285I mutant receptor was caused by decreased cell surface expression, N285I and WT receptors were tagged with a c-myc epitope (30) and used to transfect COS-7 cells, which were then subjected to flow cytometric analysis. The tagged receptors conserved their respective binding parameters (data not shown). Fig. 5 demonstrates that both the tagged WT and N285I mutant receptors were equally well expressed at the cell surface with ~30% of cells displaying receptors with similar fluorescence intensity, indicating that the loss of activation and ligand binding of the N285I mutant receptor was not due to an absence of cell surface expression.

**DISCUSSION**

PAF has numerous biological activities and stimulates multiple signaling pathways through its specific G protein-coupled receptor (13). The PAF receptor was the first G protein-coupled receptor for a lipid ligand to be cloned, and, to our knowledge, no experimental data have yet been published on residues that might be involved in lipid ligand interaction with this receptor superfamily. We have previously shown that the highly conserved Asp residue in the second transmembrane domain of the PAF receptor was not involved in direct binding of either the antagonist WEB2086 or PAF. The mutation of this residue to Asn increased affinity specifically for PAF but abolished G protein coupling (23). Our group has also demonstrated that the receptor affinity for PAF could be affected by mutations in the COOH-terminal region of the third intracellular loop of the PAF receptor (22). In the present report, we have used site-directed mutagenesis to study the possible involvement of the transmembrane residues Phe97, Phe98, Asn285, and Asp289 in ligand binding and activation of the human PAF receptor.

A conserved Asp residue is found in the third TMH of several G protein-coupled receptors and has been shown to participate in binding hydrophilic ligands such as the cationic neurotransmitters adrenaline, acetylcholine, dopamine, and serotonin (25), which are reminiscent of the positively charged PAF choline moiety. Asp residues are absent, however, from the third TMH of the PAF receptor. Thus, we mutated the two adjacent Phe residues (Phe97 and Phe98) of the third TMH into Gly residues (FFGG double mutant) to verify whether those hydrophobic amino acids, positioned at the locus of the conserved Asp residue mentioned above, were involved in ligand binding, possibly through interaction with the acetyl group or the phospholipid chain of the PAF molecule.

Our results showed that the Phe97 and Phe98 residues have no role in WEB2086 binding but, when mutated, produced a 3–4-fold decrease in affinity for PAF. This change in affinity more likely reflects an increased flexibility and, consequently, a slight conformational change of the PAF receptor rather than a modification of direct interaction of these two hydrophobic residues with the PAF molecule. This is supported by the fact that the FFGG mutant produced the same level of IPs as the WT receptor following PAF binding. It has been shown that even a small modification of either the phospholipid chain, the acetyl group, or the phosphocholine moiety of the PAF molecule resulted in considerable loss of its biological activity (2). If the Phe residues were to interact with any of the chemical groups of the PAF molecule, then the FFGG mutant activation would be significantly affected. The change in agonist affinity could be caused by the substituting Gly residues, the higher degree of freedom of movement of the third TMH, or the disappearance of hydrophobic interactions in the FFGG mutant with other residues of the receptor or with the membrane. Since the double mutant displayed only a small decrease in PAF, but not in WEB2086 affinity, these two hydrophobic residues seem to be involved in maintaining the PAF receptor in its optimal conformation. These results also suggest that the agonist and antagonist are not sensitive to the same structural variation of the receptor.

An Asn residue, corresponding to position 289 of the PAF...
Asn285 in the seventh TMH of the PAF receptor, is presumed to thromboxane A2 receptors as yet.

To our knowledge, no particular function has been attributed to the cognate Asp residues of the thrombin and TMH (33). To explain the differences between the N285A and N285I phenotypes, we might speculate that mutation of Asn285 binding. To assess the sites of interaction of these molecules with the receptor.

In summary, we have reported here and in a previous study (23) that the PAF molecule does not bind to its receptor by interacting with the Asp63, Asn285, and Asp289 residues, contrary to what had been proposed in a recent molecular model of the PAF receptor (24). Discrepancies between the molecular model and our experimental data may come from the fact that the model was based on the bacteriorhodopsin three-dimensional structure, which displays a seven-transmembrane domain topology but is not a G protein-coupled receptor and has no notable sequence homology. A projection map of rhodopsin (a G protein-coupled receptor) at a 9 Å resolution showed that the configuration of the helices of bacteriorhodopsin was different from rhodopsin (35). Such differences are likely to exist with the PAF receptor. However, we suggest that receptor conformation and transition could be influenced by the amino acids found at positions 63 (23), 97, 98, 285, and 289. Moreover, we have shown that Asp63 (23) and the residue found at position 289 are determinant in receptor activation. Work is underway in our laboratory to identify residues directly involved in ligand binding to the PAF receptor and will hopefully contribute to a better understanding of how the PAF receptor ligands bind to their receptor and effect physiological responses.

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