Two Basic Amino Acids in the Second Inner Loop of the Interleukin-8 Receptor Are Essential for Gα16 Coupling*

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The involvement of basic residues of interleukin(IL)-8 receptors in coupling to the Gi and G16 proteins was investigated by using a series of IL-8 receptor mutants. Substitution of the basic amino acids in the third inner loop of the receptor does not alter the abilities of the receptor mutants to activate recombinant Gα16 or phosphoinositide-specific phospholipase C (PLC) β2 expressed in COS-7 cells. However, an IL-8 receptor mutant with double mutations at residues Lys158 and Arg159 of the second inner loop loses its abilities to activate Gα16 but retains its ability to activate PLC β2. The activation of PLC β2 by an IL-8 receptor that is sensitive to pertussis toxin has been previously demonstrated to be mediated through Gi/γ. Surprisingly, the IL-8 receptor mutants with substitution of Ala for either residue Lys158 or Arg159 can still activate Gα16, which suggests that either of the two basic residues in the second inner loop of the IL-8 receptor is sufficient for Gα16 coupling.

Many biologically active molecules transduce their signals through specific cell-surface receptors. Some of the receptors interact with heterotrimeric GTP-binding proteins (G proteins)1 (1, 2). Molecular cloning has revealed the existence of genes encoding at least 20 G proteins in mammals (3). These subunits can form a variety of heterotrimers that serve to connect specific cell surface receptors to a large number of different effectors including at least 4 PLC β isoforms and many adenylyl cyclases, as well as several specific ion channels (1–3). One of the intriguing questions posed by this apparent complexity is how signal transduction circuits are organized so that different kinds of receptors can be connected to effectors through various G proteins and coordinate a variety of responses in a large number of different cells. The specificity of some of the circuits is determined no doubt by developmental regulation of the expression of genes that encode the receptors, G proteins and effectors. In addition, subcellular localization may contribute to the specificity to a certain extent. However, the primary determinant for formation of a specific signal transduction circuit lies in specific protein-protein interactions.

Work has been done to understand the molecular basis of the specificity in receptor-G protein interactions (4). Amino acid sequences that are involved in activation of Gαq have been mapped to the third cytoplasmic (inner) loops of the α1B-adrenergic receptor, the m1 muscarinic receptor, and the glutamate receptors by using various chimeras (5–7, 24). Although these sequences share no significant amino acid sequence homology, they appear to be different from the sequences involved in activating Gαs (8, 9). Recently, we have found that different α1B-adrenergic receptor sequences are involved in coupling to different α subunits of the Gq class (10). Furthermore, receptor sequences in other inner loops have also been implicated in the involvement of G protein coupling. Studies using receptor-derived peptides have implicated that the second inner loop of the N-formyl peptide receptor may be involved in G protein interaction (11, 12).

We have previously demonstrated that the IL-8 receptor (IL-8R), like many other chemoattractant receptors including the C5a and formyl-methionyl-leucyl-phenylalanine receptors, can couple to both G16 and Gi proteins (14). In this report, we will report our investigation of the IL-8R sequences involved in coupling to G16 but not to Gi by site-directed mutagenesis. Our results indicate that two basic amino acid residues in the second inner loop of the IL-8R are essential for coupling to Gα16 but not to Gi, whereas the basic residues in the third inner loop are not required for coupling to either Gi or G16.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum under 5% CO2 at 37 °C. The COS-7 cells were seeded the day before transfection into 24-well plates at a density of 1 × 105 cell/ml. The medium was removed the next day, and 0.5 ml of Opti-MEM (Life Technologies, Inc.), which contained 5 μg of lipofectamine (Life Technologies, Inc.) and 1 μg of plasmid DNA, was added to each well. 5 h later, the transfection medium was replaced by the culture medium. The cells were labeled with 10 μCi/ml [2-3H]myo-inositol the following day, and the levels of inositol phosphates (IPs) were determined one day later as previously described (13). All the cDNAs used in this study were constructed in the pCMV expression vector (13).

SDS-polyacrylamide Gel Electrophoresis and Western Blot—Equal numbers of transfected cells were solubilized in the SDS sample buffer and loaded to 12% SDS-polyacrylamide gels. The proteins were then electrophoresed onto nitrocellulose membranes and detected with antibody–antibodies indicated in the figure legends.

Receptor Binding Assays—COS-7 cells in 12-well plates were transfected with the cDNA encoding the IL-8R or its mutants. After 48 h, the cells were washed with phosphate-buffered saline and incubated with varying amounts of 125I-IL-8 (3000 Ci/mmol, NEN Life Science Products) in phosphate-buffered saline containing 1 mM bovine serum albumin for 1 h at 4 °C. After washing three times with ice-cold phosphate-buffered saline containing bovine serum albumin, the cells were lysed in 0.5 ml of 0.2 N NaOH, and 0.1-ml aliquots were taken for counting in a scintillation counter. The nonspecific binding was determined by measuring binding of 125I-IL-8 to nontransfected cells. The numbers of specific IL-8-binding sites (Bmax) and dissociation constants (Kd) were determined by the Scatchard analysis (24).

Construction of IL-8R Mutants—All the IL-8R mutants listed in Fig. 1 were generated by polymerase chain reaction with the high fidelity DNA polymerase, pfu (Stratagene), and from the American Heart Association (to H. J.) The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. * To whom the correspondence should be addressed. Tel.: 716-275-2029; Fax: 716-244-9283.

1 The abbreviations used are: G protein, heterotrimeric GTP-binding protein; IP, inositol phosphate; IL-8, interleukin-8; IL-8R, IL-8 receptor; PLC, phosphoinositide-specific phospholipase C; PTx, pertussis toxin.
The IL-8 receptors were previously shown to couple to two G proteins, G\(_i\) and G\(_{16}\) (14). To investigate whether different receptor sequences are involved in coupling to these two G proteins, we have generated a series of mutated receptors as tabulated in Fig. 1. Since it was postulated that the BBXXB motif might be responsible for G\(_i\) coupling (15), we first investigated whether the BBXXB motif (residues Lys\(^{247}\)-Arg\(^{251}\)) in the third intracellular loop of the human type B IL-8 receptor is involved in G\(_i\) coupling. We constructed the IL-8 receptor mutants, m1, m2, and m3, by substitution of Ala residues for the residue Lys240. The mutant m5 was substituted by an Ala residue for the residue Lys248, respectively. These mutants were tested for their abilities to couple to G\(_i\) and G\(_{16}\) in a previously established transient transfection assay (10, 14, 16–19) to characterize the G protein-coupling specificity for the IL-8 receptors. The COS-7 cells used in the assay system do not contain endogenous IL-8 receptors, PLC\(_b^2\), or Go16, although they contain Go2 and PLC\(_b^1\) (13, 14, 17, 20). Thus, IL-8 did not elicit any significant elevation of IP levels in cells expressing the wild-type receptor and its mutants in the absence of Go16 or PLC\(_b^2\) (Fig. 2A). To test the G\(_i\) coupling of the IL-8 receptor mutants, we cotransfected COS-7 cells with cDNAs encoding Go16 and the IL-8 receptor or its mutants, and IL-8-induced accumulation of IPs was determined. As shown in Fig. 2B, IL-8-induced marked PTx-resistant accumulation of IPs in cells coexpressing Go16 and the IL-8 receptor or its mutants, m1, m2, or m3, which suggests that these three IL-8 receptor mutants, like the wild-type IL-8 receptor, can still couple to Go16. To test the G\(_{16}\) coupling, we cotransfected COS-7 cells with the cDNAs encoding PLC\(_b^2\) and the receptors. The IL-8 receptor was previously shown to couple to endogenous Go proteins of COS-7 cells to release Go\(_{16}\), which then activates recombinant PLC\(_b^2\). As shown in Fig. 2C, there was clear IL-8-induced accumulation of IPs in cells coexpressing PLC\(_b^2\) and the IL-8 receptor, m1, m2, or m3, and the ligand-induced responses were mostly PTx-sensitive. Therefore, these data indicate that the IL-8 receptor mutants can couple to both Go16 and Go in transfected COS-7 cells. To test further the importance of the triple basic amino acids in the third inner loop of the IL-8 receptor, these basic amino acids (Lys\(^{246}\)-His\(^{247}\)-Arg\(^{248}\)) were mutated to three alanine residues. As shown in Fig. 2B and C, the IL-8 receptor mutant can still couple to recombinant Go16 and to PLC\(_b^2\) via endogenous Go proteins. Thus, it is clear that the BBXXB (residues Lys\(^{247}\)-Arg\(^{251}\)) motif at the N-terminal end of the third intracellular loop of the IL-8 receptor is by no means involved in the Gi coupling or the G\(_{16}\) coupling.

Another basic amino acid residue in the third inner loop, Lys\(^{246}\), was also investigated for its involvement in coupling to Go16 or Go. We constructed the mutant m5 by substitution of an Ala residue for the residue Lys\(^{240}\). The mutant m5 was subjected to the same tests as m1–4. The tests showed that m5, like the others, can couple to Go16 and Go. Thus, we conclude that the basic residues inside the third inner loop of the human type B IL-8 receptor are not involved in coupling to Go16 or Go.

Search of the IL-8 receptor sequence revealed a BBXXXB (Lys\(^{158}\)-Lys\(^{163}\)) motif in the second inner loop of the receptor.

**RESULTS AND DISCUSSION**

FIG. 1. Summary of IL-8R mutant constructs, G protein coupling, and ligand-binding properties. The amino acid sequences of the second and third inner loops of IL-8R are shown. Designations of IL-8R mutants and mutations in each of the IL-8R mutants are also elucidated. Data regarding the G protein coupling are shown in Figs. 2 and 3. Ligand binding was determined as described under “Experimental Procedures.” The unit for B\(_{\text{max}}\) is fmol/10\(^5\) cells.
RESULTS

1. To test whether the basic residue doublet (Lys<sup>158</sup>-Arg<sup>159</sup>) is involved in the G protein coupling, we replaced the doublet with two Ala residues creating the mutant m8 (Fig. 1). By testing the mutant in the cotransfection assay, we found that m8 can induce IP<sub>3</sub> accumulation only in cells coexpressing PLC β2 (Fig. 3B) but not in those coexpressing Ga16 (Fig. 3A), which suggests that m8 can couple only to Gi but not to Ga16. Neither m6 nor m7, which have substitution of an Ala residue for one of the basic residue doublets, loses its ability to couple to Ga16 (Fig. 3). The ability of m8 to activate PLC β2 has eliminated the possibility that the mutations in m8 greatly changed the conformation of the receptor. Nevertheless, we also did the ligand-binding assay with<sup>125</sup>I-IL-8. The expression level of m8 and its affinity for IL-8 are similar to those of the wild-type IL-8 receptor, m6 and m7 (Fig. 1). In addition, we also determined the expression levels of Ga16 in cells coexpressing m8, m6, m7 and the wild-type IL-8 receptor. No major differences were noticed (Fig. 3C). Therefore, it is reasonable to conclude that either of the basic residues (Lys<sup>158</sup> and Arg<sup>159</sup>) is apparently sufficient to retain the ability of the receptor to couple to Gi16 and that the presence of either of them is essential for the G16 coupling, although these two residues do not appear to play a significant role in the Gi coupling.

We have previously demonstrated that different α1-adrenergic receptor sequences are involved in coupling to Goq/11 and Ga14. However, sequences involved in Ga16 coupling have not been elucidated. Recent reports (18, 21) show that Ga16 appears to be promiscuous in its coupling to various receptors. Almost all of the G protein-coupled receptors thus far tested, including Gq-, Gi-, and Gs-coupling receptors, can couple to Ga16 in transfected COS-7 cells (18, 21). This coupling promiscuity suggests that most G protein-coupling receptors possess the sequence elements and/or conformation required for interaction with and activation of Ga16. Our results provide an insight into what the requirements are. The basic residues Lys<sup>158</sup> and Arg<sup>159</sup> may constitute the sequence that interacts with and activates Ga16 or may be critical for formation of the receptor conformation required for coupling with Ga16. More studies (knowledge of the three-dimensional structure of the receptor) are needed to understand exactly how these two basic residues are involved in Ga16 coupling. Our data also indicate that the BBXXB motif in the third loop of IL-8R is not essential for either Goi or Go16 coupling. These data are consistent with the observation that residue Met<sup>241</sup> in the third loop, as well as other non-charged amino acid residues in the second loop of IL-8R, are involved in coupling to Goi2 (22).

Receptor consensus sequences for G protein-coupling were being pursued vigorously in the past. No such sequences have, however, been identified. Therefore, it is now generally believed that each individual receptor possesses specific receptor coupling elements, which were mostly found in the third inner loops of various receptors. Ga16 is an intriguing subunit. It lacks receptor coupling specificity; it couples to various G protein-coupling receptors ranging from Gs to Gi and Gq-coupling receptors. We have been looking for the receptor elements that are required for Ga16 coupling in both α1B-adrenergic receptors (10), but these elements have been eluding us until we identified the dual basic amino acids in the second loop of the IL-8 receptor. Although we did not identify consensus sequences for G16 coupling, our results are of great significance. 1) These results unequivocally prove that the second loop is involved in G protein-coupling specificity in contrast to most other studies, which usually only implicate the third inner loops. 2) This is the first time that Ga16-coupling elements have been identified. 3) The element required for Ga16 coupling is not required for Gqi coupling. 4) The basic residues in the second and third inner loops, which have been widely believed to be involved in Gi coupling, are not important for Gi coupling by the IL-8 receptor. Therefore, this work provides us with a better understanding of the specific interactions between receptors and G proteins. In addition, the receptor mutants that show limited yet defined G protein-coupling specificity would be useful in determining the specific in vivo function of signal transduction pathways mediated by specific receptors and G proteins.

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IL-8 Receptor and G Protein Coupling

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