The First Inner Loop of Endothelin Receptor Type B Is Necessary for Specific Coupling to Ga13

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Endothelin (EDN) receptor type B (EDNRB) activates serum response factor (SRF) via Gq11 and G12/13 G proteins. In this study, we investigated the involvement of intracellular loop sequences of EDNRB in coupling to these G proteins. EDNRB mutants were generated and tested for their abilities to activate SRF in NIH3T3 cells and in the mouse embryonic fibroblast cell line (Fq/11) lacking both Gaq and G11. EDNRB can activate SRF in NIH3T3 cells via Gq11, although it can only activate SRF through G12/13 in Fq/11 cells. Mutants with mutations in the second and third inner loops of EDNRB functioned in the same manner in both cell lines, either able or unable to activate SRF. This finding suggests that the second and third inner loops of EDNRB either participate or not in coupling to both Gq11 and G12/13 but are not specific for either one. However, in the first inner loop, a substitution of three Ala residues for Met128, Arg129,Asn130 abolished the ability to activate SRF only in Fq/11 cells, suggesting that this mutation might specifically disrupt the coupling to G12/13 rather than to Gq11. Further characterization of this first inner loop mutant revealed that exogenous expression of Gaq or G13 could restore SRF activation, whereas the expression of G13 did not. Therefore, we conclude that although the three intracellular loops of EDNRB may be involved in coupling to G proteins, residues Met128,Arg129,Asn130 in the first intracellular loop are specifically required for activation of Ga13.

Endothelin (EDN)1 is a potent vasoactive peptide, which can induce a wide range of cellular and physiological responses (1). In mammalian cells, there are at least two EDN receptor subtypes, EDNR and EDNRB. The EDN receptors belong to the superfamily of rhodopsin-like G protein-coupled receptors (GPCRs) that contain seven transmembrane domains. The extracellular and transmembrane domains of GPCRs are involved in ligand binding, whereas the intracellular domains are involved in G protein coupling and subsequent effector regulation.

Both EDN receptors can couple to Gq proteins, resulting in the activation of phospholipase Cβ (2). This pathway was shown to be necessary for EDN-induced activation of the ERK/MAPK signaling cascade (3). In addition, EDN receptors were also found to regulate adenyl cyclases through both Gaq and G13 (2). More recently, EDN receptors were demonstrated to couple to the G13 subfamily of G proteins that consists of G12 and G13 (4). In addition, EDNRB was shown to induce stress fiber formation via G13 in fibroblast cells (5), whereas EDNRB was shown to couple to Ga13 in human HEK-293 cells (6).

Because many GPCRs can couple to more than one Go subunit, understanding the structural basis for specificity between a receptor and its respective G proteins is an area of active investigation (7, 8). For example, the amino acid sequences involved in Go activation have been mapped to the third inner loops of the β2-adrenergic receptor, the m1 muscarinic receptor, and the glutamate receptors (9–11). Our own work has identified two basic amino acids in the second inner loop of the interleukin-8 receptor that is required for Go16 coupling (12). The structural specificity of G protein coupling has also been demonstrated in EDNRs. Studies using various receptor chimeras demonstrate that the second inner loop of EDNRA is involved in Go coupling, whereas the third inner loop of EDNRB receptor is responsible for Gaq coupling (13). In addition, EDNRs are modified by palmitoylation at a cluster of cysteine residues in the C-terminal tails, which also appear to play a role in G protein coupling (14).

In this study, we investigated the molecular basis of Ga12/13 coupling to EDNRB. Taking advantage of a previously established reporter gene assay system in NIH3T3 and Fq/11 cells (derived from embryos in which the genes for Gaq and G11 were disrupted) (4, 15), we identified three residues in the first inner loop of EDNRB as required for specific activation of Ga13.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Luciferase Assay—NIH3T3 and Fq/11 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37 °C under 5% CO2. The Fq/11 cell line was established from mice lacking both Gaq and G11 (6). For transfection, cells (5×104 cells/well) were seeded into 24-well plates the day before transfection. Cells were transfected with 0.5 μg of DNA/well using LipofectAMINE Plus (Invitrogen) as suggested by the manufacturer. Transfection was stopped after 3 h by switching to Dulbecco’s modified Eagle’s medium containing 0.5% fetal bovine serum. Cell extracts were collected 30 h later for luciferase assays.

Luciferase assays were performed using a Constant Light luciferase assay kit (Roche Molecular Biochemicals) as instructed. Cell lysates were first determined for the fluorescence intensity emitted by GFP proteins in a Wallac Victor2 Multicounter (PerkinElmer Life Sciences) followed by luminescence measurement by using the same counter after the addition of the luciferase substrate. Luminescence intensities were normalized against fluorescence intensities as described previously (4).

Phosphatidic Cq Assay—Cells were incubated with 10 μCi/ml of myo-[3-3H]inositol in 0.25 ml of insulin-free Dulbecco’s modified Eagle’s medium containing 10% dialyzed fetal bovine serum at 37 °C under 5% CO2 until the incorporation of radioactivity was linear. The costs of publication of this article were defrayed in part by advertisement "in accordance with 18 U.S.C. Section 1734 marked "(

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6 The abbreviations used are: EDN, endothelin; EDNR, endothelin receptor; Fq/11, a mouse embryonic fibroblast cell lines derived from mice lacking Gaq and G11; GPCR, G protein-coupled receptor; GFP, green fluorescent protein; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; SRF, serum response factor.
the cells were lysed in 0.5 ml of 0.2N NaOH and 0.1-ml aliquots were
ice-cold phosphate-buffered saline containing bovine serum albumin,
SRF via coupling to both Gq/11 and G12/13 proteins in NIH3T3
previous studies have demonstrated that EDNRB activated
in Fig. 1A with deletions in the third inner loop of EDNRB as illustrated
–11, 20), we first tested receptor mutants
protein coupling (7–11, 20), we first tested receptor mutants
inner loop of GPCRs were frequently found to be involved in G
Because previous work has found that residues in the third
mutants were lysed in the SDS-PAGE sample buffer and incubated at
37 °C for 24 h. The levels of inositol phosphates were determined as
determined by measuring the binding of [125I]EDN1 to cells transfected
the Scatchard analysis.
Construction of EDNRB Mutants—All EDNRB mutants were generated
polymerase chain reaction with the high fidelity DNA Pfu
polymerase (Stratagene), and each of the mutations was confirmed by
DNA sequencing and restriction digestion. Hemagglutinin epitope tags
were incorporated at the C termini of EDNRB and its mutants.
Western Analysis—NIH3T3 cells expressing LacZ, ENDRB, or its
mutants were lysed in the SDS-PAGE sample buffer and incubated at
37 °C for 30 min before electrophoretic separation in 10% acrylamide
gels. After electrophoresis, proteins were blotted to nitrocellular mem-
resulting from the deletion of the third inner loop of GPCRs, the third
loop of GPCRs were frequently found to be involved in G
Because previous work has found that residues in the third
CO2 for 24 h. The levels of inositol phosphates were determined as
described previously (17).
   
   Ligand Binding Assay—NIH3T3 or Fq/11 cells in 24-well plates were
transfected with the cDNAs encoding the EDNRB or its mutants. After
24 h, the cells were washed with phosphate-buffered saline and incu-
bated with varying amounts of [125I]EDN1 (3000 Ci/mmol, PerkinElmer
Life Sciences) in 200 μl of phosphate-buffered saline containing 1 mg/ml
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 γ-ray counter. The nonspecific binding was
determined by measuring the binding of [125I]EDN1 to cells transfected with
the LacZ expression plasmid. The number of specific EDN1 bind-
ing sites (Bmax) and dissociation constants (KD) were calculated
using the Scatchard analysis.
   
   Construction of EDNRB Mutants—All EDNRB mutants were generated
by polymerase chain reaction with the high fidelity DNA Pfu
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mutants were lysed in the SDS-PAGE sample buffer and incubated at
37 °C for 30 min before electrophoretic separation in 10% acrylamide
gels. After electrophoresis, proteins were blotted to nitrocellular mem-
and probed with an antibody to the hemagglutinin tags carried by these recombinant proteins.

RESULTS AND DISCUSSION

Previously, we demonstrated that EDNRB could couple to
G12/13 (4). To further understand the interaction between ED-
NRB and G12/13, we generated a series of receptor mutants in
which the intracellular loop regions were mutated and tested for
their ability to activate SRF in NIH3T3 and Fq/11 cells. Because previous work has found that residues in the third
inner loop of GPCRs were frequently found to be involved in G
protein coupling (7–11, 20), we first tested receptor mutants
with deletions in the third inner loop of EDNRB as illustrated in
Fig. 1A for their ability to activate SRF (Fig. 1, B and C). Our
previous studies have demonstrated that EDNRB activated SRF via coupling to both Gq/11 and G12/13 proteins in NIH3T3
cells, whereas EDNRB activated SRF via G13 and/or G13 in
Fq/11 cells (4). As shown in Fig. 1, B and C, EDN1 could activate SRF in NIH3T3 or Fq/11 cells expressing mt1, mt2, mt4, or mt6
(Fig. 1, B and C) but not in cells expressing mt3 or mt5. Because the mutation in mt5, which covers the mutation in
mt3, did not affect ligand-binding characteristics (Table I), we
conclude that the amino acids deleted in mt5 are probably
required for coupling to both Gq/11 and G13. In addition, the
sequences deleted in mt1, mt2, mt4, and mt6 do not appear to
be essential for coupling to either Gq/11 or G13.

We went on generating two additional mutants that contain
deletions spanning the short second inner loop (Fig. 2A).
Both mutants failed to activate SRF in response to EDN1 in NIH3T3
cells (Fig. 2B). Given that neither of the mutations affected
ligand binding (Table I), it is probable that these mutations
knock out coupling to multiple G proteins. We also made a
C-terminal deletion mutant with a deletion up to residue

![Fig. 1. Effects of mutations in the third inner loop of EDNRB on G protein coupling. A, schematic representation of the third inner loop of EDNRB and its mutants. Designation for the EDNRB mutants and positions of the deletion mutations are shown. Expression of EDNRB and its mutants in NIH3T3 cells were analyzed by Western blotting using an antibody to the hemagglutinin tags carried by these recombinant proteins. B, regulation of SRF in NIH3T3 cells. NIH3T3 cells were transfected with 0.1 μg of serum response element-luciferase reporter plasmid, 0.1 μg of wild type EDNRB or one of the mutants (mt1–6). The next day, cells were lysed 6 h after the addition of EDN1 (2 nm). GFP levels and luciferase activities were determined. The luciferase activities presented are normalized against the levels of GFP expression. Experiments were carried out in duplicates and repeated at least three times. The representative experiments are shown. The levels of luciferase activity in the absence of ligand are ~2000 arbitrary units (AU). C, regulation of SRF in Fq/11 cells. The Gq/11-deficient cells were transfected as in B. The data collection and analyses were carried out as described in B. The levels of luciferase activity in the absence of ligand are ~2500 AU.](https://www.jbc.org/content/285/39/2385/F1.large.jpg)

![Fig. 2. Effects of mutations in the second inner loop of EDNRB on G protein coupling. A, schematic representations of the second inner loop of EDNRB and its mutants. B, SRF regulation. NIH3T3 cells were transfected and treated as described in Fig. 1B.](https://www.jbc.org/content/285/39/2385/F2.large.jpg)
Cys<sup>405</sup>, a myristoylation site. This C-terminal deletion mutant showed increased responses to EDN1 in both 3T3 and Fq/11 cells (data not shown), probably because of the removal of desensitization signals localized at the C-terminal tail of the receptor as found with many other GPCRs (16, 21).

Thus far, specific mutations in the second and third inner loops of EDNRB have functioned in a similar manner in both cell lines. This suggests that although certain regions of the second and third inner loops are essential for G protein coupling, they are not specific for any particular G protein. Therefore, we shifted our focus to the first inner loop. In this segment, there are two sets of triple amino acids that contain basic or polar residues. We mutated these sites with three Ala residues (Met<sub>128</sub>-Arg<sub>129</sub>-Asn<sub>130</sub>) in the first inner loop of EDNRB. The binding of [<sup>125</sup>I]END1 to the cells were carried out as described under “Experimental Procedures.” Results are shown after subtracting the nonspecific binding, which is the binding to cells transfected with the LacZ expression plasmid.

**TABLE II**

|        |<sup>K<sub>d</sup></sup> (nM) |<sup>B<sub>max</sub></sup> (receptors/cell) × 10<sup>-4</sup> |
|--------|----------------|-------------------|
| WT     | 0.19            | 7.2               |
| mt10   | 0.21            | 11                |

**Fig. 3.** Effects of mutations in the first inner loop of EDNRB on G protein coupling. A, schematic representations of third inner loop mutations of two EDNRB mutants and Western analysis of their expression. B and C, SRF regulation in NIH3T3 cells and Fq/11 cells. NIH3T3 cells and Fq/11 cells were transfected and treated as described in Fig. 1.

**Fig. 4.** Specific coupling of EDNRB mutant mt10 to G<sub>13</sub>, A and B, SRF regulation. NIH3T3 and Fq/11 cells were transfected as described in Fig. 1. The next day, the cells were treated for 6 h with two doses of EDN1 (0.5 and 5 nM). The next day, luciferase activity was determined 6 h after the addition of EDN1 (0.5 nM). At the present transfection condition, G protein when expressed by itself showed little activation of the reporter system in Fq/11 cells.

**Fig. 5.** Binding of [<sup>125</sup>I]EDN1 to NIH3T3 cells. NIH3T3 cells were transfected with plasmids expressing LacZ, the wild type, or mt10 EDNRB. The binding of [<sup>125</sup>I]EDN1 to the cells were carried out as described under “Experimental Procedures.” Results are shown after subtracting the nonspecific binding, which is the binding to cells transfected with the LacZ expression plasmid.

Response to EDN1 (Fig. 4C) when G<sub>13</sub> was expressed, confirming that this mutant receptor is unable to couple to G<sub>13</sub>. Interestingly, when G<sub>q12</sub> was expressed, EDN1-induced SRF activation was restored (Fig. 4C), suggesting that residues Met<sup>128</sup>-Arg<sup>129</sup>-Asn<sup>130</sup> are required for specific coupling to G<sub>13</sub> but not to G<sub>q12</sub>. The reason for the failure of activation of SRF in the absence of transfected G<sub>q12</sub> might be because of a low or no expression of endogenous G<sub>q12</sub> in these embryonic cells. As anticipated, the expression of G<sub>q</sub> also restored the ability of mt10 to activate SRF in response to EDN1 (Fig. 4C), confirming that mt10 can couple to G<sub>q12</sub>. The ability of mt10 to couple to the G<sub>q</sub> proteins was also validated by the finding that EDN1 could stimulate the accumulation of inositol phosphates in NIH3T3 cells expressing mt10 (data not shown). Moreover, the ability of the wild type receptor to increase reporter gene activity in the presence exogenous G<sub>q13</sub>, G<sub>q12</sub>, or G<sub>q6</sub> in Fq/11 cells indicates that ENDNRB is capable of coupling G<sub>q13</sub> and that the inability of mt10 to activate SRF in the presence of exogenous G<sub>q13</sub> is not because of insufficient expression of G<sub>q13</sub> in the Fq/11 cells. Furthermore, ligand-binding analyses, which were carried out in both NIH3T3 and Fq/11 cells (Fig. 5 and Tables I and II), did not reveal obvious differences between mt10 and the wild type receptor in the values of both B<sub>max</sub> and K<sub>d</sub>, suggesting that mt10 was expressed normally at the cell surfaces of both NIH3T3 and Fq/11 cells. Putting all of these results together, we conclude that residues Met<sup>128</sup>-Arg<sup>129</sup>-Asn<sup>130</sup> in the first inner
loop of EDNRB are involved in specific coupling to \( \text{G}_{13} \) but not to \( \text{G}_{12} \) or \( \text{G}_{q} \).

The structural determinants of receptor G protein coupling have been pursued vigorously in the past. Although no consensus sequences have been identified, specific sequences in individual receptors have been identified for coupling to distinct G proteins (7, 8). The coupling elements are often found in the third inner loops of various GPCRs with a few exceptions. For instance, the coupling of the interleukin-8 receptor to \( \text{G}_{12} \) and the EDNRA receptor to \( \text{G}_{q} \) was mapped to the second inner loop (13, 15). As for EDNRB, different elements had previously been found to be required for coupling to \( \text{G}_{q} \) and \( \text{G}_{13} \) proteins (13). In this report, we demonstrate for the first time that the first inner loop of a GPCR is required for specific coupling to a G protein.

Although distinct sequence elements have been found for specific coupling to different G proteins, evidence also suggests that there may be multiple contacts made between a GPCR and a given G protein. It appears that there might also be some common sequences that are required for coupling to different G proteins. For example, the sequences deleted in mt5, mt7, and mt8 appeared to be required for coupling to both \( \text{G}_{13} \) and \( \text{G}_{q} \) proteins. However, although we have demonstrated that these mutations do not affect ligand-binding characteristics, we cannot eliminate the possibility that the mutations disrupt certain structures formed by the intracellular loops, which rather than the sequences themselves are required for G protein coupling. Nevertheless, the third inner loop sequences of many GPCRs, particularly those near the sixth transmembrane domain, appear to be critical in coupling to \( \text{G}_{q} \) and \( \text{G}_{13} \) (7, 8). In this report, we added \( \text{G}_{13} \) to that list.

Identification of mutations that can specifically knock out the coupling of EDNRB to \( \text{G}_{13} \) lays the groundwork for future investigations of specific physiological significance of the EDNRB-\( \text{G}_{13} \) signaling pathway. The importance of EDN in the cardiovascular system has been well documented (1). Not long ago, \( \text{G}_{13} \) was also found to play an important role in vasculature regulation because mice lacking \( \text{G}_{13} \) died from vasculature malfunction (18). In addition, the inactivation of the EDNRB gene produced a megacolon associated with spotted coat color in mice (19). Similar phenotypes are also manifested in patients with Hirschsprung’s disease. It would be very interesting to determine whether the EDNRB-\( \text{G}_{13} \) signaling pathway is involved in these processes. Our finding provides a potential tool for such an investigation.

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