The Inducible G Protein-coupled Receptor edg-1 Signals via the G\textsubscript{i}/Mitogen-activated Protein Kinase Pathway*

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The edg-1 gene encodes an inducible G protein-coupled receptor (GPR) homologue that is induced during the in vitro differentiation of human endothelial cells. The aim of this study was to investigate the G protein-coupling and -signaling properties of the edg-1 polypeptide. The third cytosolic loop (i3) of edg-1 associates with Go\textsubscript{a} and Go\textsubscript{o} polypeptides in a guanosine 5'-O-(thiotriphosphate)-sensitive manner. Immunoprecipitation of the edg-1 polypeptide in transfected cells results in the co-precipitation of Go\textsubscript{a} and Go\textsubscript{o} polypeptides. These data strongly suggest that edg-1 is capable of coupling to the G\textsubscript{i} pathway. Overexpression of the edg-1 GPR in human embryonic kidney 293 cells results in the sustained activation of the MAP kinase activity that is blocked by pertussis toxin treatment. Moreover, NIH3T3 cells permanently transfected with edg-1 exhibit enhanced MAP kinase and phospholipase A2 activities. These data suggest that the G\textsubscript{i}/mitogen-activated protein kinase pathway is a major signaling pathway regulated by the orphan receptor edg-1.

Angiogenesis, new blood vessel formation, is a critical component of many physiological processes such as wound healing and development. Uncontrolled angiogenesis is associated with numerous pathological conditions, including diabetic retinopathy, rheumatoid arthritis, and solid tumor growth (1). The third cytosolic loop (i3) of edg-1 associates with Go\textsubscript{a} and Go\textsubscript{o} polypeptides in a guanosine 5'-O-(thiotriphosphate)-sensitive manner. Immunospecific immunoprecipitation of the edg-1 polypeptide in transfected cells results in the co-precipitation of Go\textsubscript{a} and Go\textsubscript{o} polypeptides. These data strongly suggest that edg-1 is capable of coupling to the Gi pathway. Overexpression of the edg-1 GPR in human embryonic kidney 293 cells results in the sustained activation of the MAP kinase activity that is blocked by pertussis toxin treatment. Moreover, NIH3T3 cells permanently transfected with edg-1 exhibit enhanced MAP kinase and phospholipase A2 activities. These data suggest that the Gi/mitogen-activated protein kinase pathway is a major signaling pathway regulated by the orphan receptor edg-1.

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An important step in the characterization of a GPR is to identify the associated G protein(s). Such information may help reveal the downstream signaling pathways. Three approaches have been used to determine which G protein subtypes can couple to a specific GPR. Kurose et al. (14) reconstituted purified α2a- and α2b-adrenergic receptors with recombinant Gi subunits in phospholipid vesicles and measured the ability of agonists to stimulate GTPase activity. Second, specific Gi subunits have been immunoprecipitated from cellular extracts and the presence of co-precipitated GPR was measured by ligand binding assays (15). Third, co-transfection of receptors and specific G protein subunit cDNAs into receptor negative cells followed by the measurement of ligand-activated second messenger pathways have been used (16). The principal requirement of each of these approaches is the knowledge of agonists for the GPRs. However, in the case of edg-1, the ligands or agonists/antagonists are unknown at present. Thus, edg-1 is an example of a putative GPR, based solely on sequence similarity with the known GPR superfamily. We therefore explored other methodologies for examining the signaling properties of edg-1.

Previous studies have shown that the third intracellular loop (i3) of GPRs are important for G protein interaction and signal transduction (reviewed in Ref. 17). Moreover, structural characteristics of Gi contact sites on the receptors have been predicted to form amphipathic α-helices (17). The i3 domain of edg-1 fits this structural model as determined by Helicalwheel analysis (18). Therefore, we constructed a fusion protein of i3...
and glutathione S-transferase (GST-i3) and utilized it as an affinity matrix to study G protein interaction in vitro. In this report, we show that Gαo and Goq associate with the GST-i3 fusion protein in vitro. Furthermore, we demonstrate that intact edg-1 binds to Gαo and signals via the G1 pathway to regulate cellular MAP kinase activity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—HUVEC (passage 4–14) were grown on fibronectin-coated plates in Medium in 199 (Mediata) supplemented with 10% fetal bovine serum (Hyclone), antibiotic and antimycotic mixture (1x RH Bioscience), 150 mM glucose, and endothelial cell growth factor. Human umbilical vein endothelial cells (HUVECs) (passage 4–14) were grown on fibronectin-coated plates in Medium in 199 (Mediata) supplemented with 10% fetal bovine serum, antibiotic, and antimycotic mixture.

**Construction and Isolation of GST-i3 Fusion Protein—DNA fragment corresponding to the third cystolic loop (l3) of edg-1 was amplified by the polymerase chain reaction (PCR) and subcloned into pGEX-2T (Pharmacia Biotech Inc.). The primers used were 1') 5'-ATG GAT CCA GAA TCT ACT CCT TGG TGA CTA CT-3' (sense) and 2') 5'-TAC CCG GGT TAC TAT AGC AGC GCC GAC GTC TCC-3' (antisense). The resulting clones were confirmed by DNA sequencing. The fusion protein was purified by glutathione-Sepharose 4B (Pharmacia) affinity chromatography using the Samplexel solution extraction method (19).

**Cellular Protein Extraction and G Binding Analysis—HUVEC cells were extracted with 1 ml of buffer A (50 mM Tris pH 7.8, 1 mM EDTA, 5 mM MgCl2, 20 mM CHAPS, 20% glycerol, 10 μg of both aprotinin and leupeptin, and 20 μg of 50 mM phenylmethylsulfonfluryl fluoride). The sample was extracted on ice for 45 min with constant stirring and centrifuged at 23,000 × g for 15 min at 4°C. The supernatant was collected, and the protein concentration was determined by Bradford method (Bio-Rad).

**To perform the in vitro binding assay, 750 μg of HUVEC extracts were incubated with 15 μg of GST fusion protein beads (approximately 10 μl of GST-i3 fusion protein bound to 165 μl of 75% glutathione-Sepharose slurry) for 2 h at 4°C. Beads were then washed three times with phosphate-buffered saline. Subsequently, bound GST subunits were detected by [35S]PAP-digestion with pertussis or cholera toxins. In vitro ADP-ribosylation reactions were carried out essentially as described (20).

**For Western analysis, G protein complexes bound to GST-i3 or GST-glutathione Sepharose 4B beads were separated by SDS-PAGE and transferred to nitrocellulose paper. Proteins were then transferred to nitrocellulose paper, blocked with btdo (5% nonfat dried milk, 50 mM Tris-HCl (pH 8.0), 2 mM CaCl2, 80 μM NaCl, 0.02% NaN3, and 0.2% Nonidet P-40) for 1 h at room temperature and blotted (5% nonfat dried milk, 50 mM Tris-HCl, pH 6.8, 0.01% (w/v) b thrombomilen blue). The [35S]PAP-labeled proteins were then subjected to SDS-PAGE on 10% polyacrylamide gels, and autoradiographed.

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RESULTS

Pertussis Toxin-sensitive Gα Subunits Associate with the GST-i3 Fusion Protein—In order to characterize the G proteins that are coupled to edg-1, the GST-i3 fusion protein was constructed and purified by affinity chromatography on glutathione-Sepharose. Coomassie Blue staining of purified GST-i3 (32 kDa) and GST (28 kDa) is shown. C, HUVEC extracts were either directly ADP-ribosylated (lanes 1–3), or affinity-isolated with GST control protein (lanes 4–6) or GST-i3 (lanes 7–9) followed by ADP-ribosylation as described under “Experimental Procedures.” Autoradiographs of protein complexes subjected to [32P]ADP-ribosylation in the absence of toxin (lanes 1, 4, and 7), or presence of pertussis toxin (PTX) (lanes 2, 5, and 8) or cholera toxin (CTX) (lanes 3, 6, and 9) are shown. These results are representative of two independent experiments.

Gαi and Gαo Associate with GST-i3 Fusion Protein in Vitro—We next examined whether Gαi1, Gαo, or both are capable of interacting with GST-i3. This was addressed by immunoblot analysis of the GST-i3 associated polypeptides with anti-Gαi1, anti-Gαo antibodies. As shown in Fig. 2A, both anti-Gαi1/Gαo antibodies react with the 41-kDa protein band from the GST-i3 affinity isolates. This association was specific because Gαi subunits did not bind to the GST control polypeptide. We also evaluated the nucleotide dependence of this in vitro interaction. HUVEC extracts were preincubated with the non-hydrolyzable analog of GTP (GTPγS) and the interaction of Gαi subunits and GST-i3 fusion protein was assessed. As shown in Fig. 2B, GTPγS treatment dramatically reduced GST-i3-associated the Gαi subunits, indicating that the interaction between Gαi subunits and edg-1-i3 is dependent on the nucleotide-bound state of the G protein.

To determine the G protein specificity of GST-i3, specific Gα subunits were expressed in HEK293 cells by transient transfection. Cell extracts were prepared and analyzed for their ability to bind to GST-i3. As shown in Fig. 3, immunoblot analysis with subunit-specific antisera show that all three Gα subunits as well as the Gα1 subunit associated with the GST-i3 fusion protein but not with the GST control protein. These data suggest that the i3 domain of edg-1 is capable of binding to the G family and Gα polypeptides in vitro.

Direct Interaction of edg-1-i3 Domain with Gα Polypeptide in the Two-hybrid Assay—Since the in vitro binding assays described above do not distinguish between direct binding versus association in a multicomponent complex, we utilized the two-hybrid interaction system to determine if the i3 polypeptide is capable of directly binding to the Gα2 polypeptide. The edg-1-i3 domain was cloned as a C-terminal fusion with the DNA binding domain of GAL4 (GAL4-i3). In addition, the myristoylation site deleted form of the Gα2 polypeptide was fused with the activation domain of GAL4 (Act D-Gα2). The resulting transformants were plated on selective media and the colonies were assayed for β-galactosidase activity. While the single plasmid transformants did not transactivate the β-galactosidase reporter gene, co-expression of GAL4-i3 and Act D-Gα2 resulted in strong transactivation, indicating direct interaction between the two proteins (Table I). Co-transformation of the GAL4-i3...
therefore examined the ability of Gi
sufficiently transfected with the epitope-tagged
immunoprecipitated with the anti-M2 monoclonal antibody
further discriminate the coupling of
plasmid with irrelevant controls such as FGF-1 fused to the
GAL4 activation domain produced colonies which were nega-
tive (white) for
mental Procedures." The expression of transfected polypeptides were
Cultured HEK293 cells were transiently transfected with
edg-1 and G\textsubscript{\alpha} interact in the yeast
two-hybrid assay
Table I

| DNA binding domain | Activation domain | Color | \(\beta\)-Gal Activity (RLU/\textmu g/15 min) |
|--------------------|------------------|-------|---------------------------------------|
| GAL-4-i\textsubscript{3} | Act D-G\textsubscript{\alpha} | White | 1.67 ± 1.5 |
| GAL-4-i\textsubscript{3} | Act D-G\textsubscript{\alpha} | White | 9.8 ± 3.0 |
| GAL-4-FGF | Act D-G\textsubscript{\alpha} | White | 4.63 ± 0.58 |
| GAL-4-FGF | Act D-G\textsubscript{\alpha} | Blue  | 3.72 ± 1.5 |
| GAL-4-i\textsubscript{3} | Act D-G\textsubscript{\alpha} | Blue  | 5107 ± 47 |

Fig. 4. Co-immunoprecipitation of epitope-tagged full-length
edg-1 with G\textsubscript{\alpha} and G\textsubscript{\alpha}2. HEK293 cells were transiently transfected with the
FLAG epitope-tagged full-length edg-1 and individual G\textsubscript{\alpha}
dNAS and cellular extracts were prepared as described under "Exper-
tmental Procedures." The expression of transfected polypeptides were
detected by Western blot analysis with the M2 antibody (FLAG-edg-1),
G\textsubscript{\alpha}1/G\textsubscript{\alpha}2 and G\textsubscript{\alpha}2/G\textsubscript{\alpha}3, antisera. B, cellular extracts from co-transfected
HEK293 cells were immunoprecipitated with anti-M2 antibody and the
G\textsubscript{\alpha} subunits present in the immunoprecipitates were detected by West-
ern blot analysis with G\textsubscript{\alpha}1/G\textsubscript{\alpha}2 or G\textsubscript{\alpha}2/G\textsubscript{\alpha}3 antisera in respective lanes.

Toxin, labeled G\textsubscript{\alpha} polypeptides were only observed in the cells
co-transfected with edg-1 and either G\textsubscript{\alpha}1 or G\textsubscript{\alpha}2 (data not shown).
No [\textsuperscript{32}P]ADP-labeled G\textsubscript{\alpha} was detected in the presence of choleratoxin (data not shown), indicating that G\textsubscript{\alpha} polypep-
tides are unable to couple to intact edg-1. These data strongly
suggest that G\textsubscript{\alpha}1 and G\textsubscript{\alpha}3 interact with intact edg-1 in trans-
sected HEK293 cells.

Activation of MAP Kinase by Edg-1. Overexpression—Re-
cently, Lefkowitz and co-workers (33) proposed a "two-state
model" of receptor activation, in which receptors are in dy-
namic equilibrium between inactive and G-protein coupled,
spontaneously active conformations. Therefore, overexpression
of receptors results in a significant increase in the population
of constitutively active receptors (34). Analogously, overex-
pression of G\textsubscript{\alpha} and its cognate receptors induced the basal activity
of signaling pathways in the absence of agonists (35). Because
G\textsubscript{\alpha}-coupled receptors are known to modulate signaling path-
ways such as the MAP kinase pathway (36), we overexpressed
edg-1 in HEK293 cells and measured the cellular MAP kinase
activity.

Cultured HEK293 cells were transiently transfected with
edg-1. MAP kinase activity was assayed by an in-gel kinase
assay of extracellular signal regulated kinase-2 (ERK-2) immu-
noprecipitates. As shown in Fig. 5A, immunoprecipitation of
HEK293 cells with the ERK-2 antiserum followed by in-gel
kinase assay in MBP-impregnated gels detected a specific band at 42 kDa. The kinase activity of this band is strongly (approximately 6-fold) induced by treatment with PMA for 15 min (data not shown). This result indicates that the 42-kDa band is ERK-2. Transient transfection of the edg-1 expression vector into HEK293 cells, in a dose-dependent manner, induced the ERK-2 kinase activity (Fig. 5B). The ERK-2 MAP kinase activity was induced at 30–60 h post-transfection and thus appears to be of sustained kinetics. Pertussis toxin treatment blocked the edg-1-dependent MAP kinase activation. The inhibition of ERK-2 activation in edg-1-transfected cells by pertussis toxin is not due to the inhibition of transfected gene expression as indicated in the M2 immunoblots. These data indicate that edg-1 signaling via the G_i pathway is responsible for sustained activation of the MAP kinase pathway.

To further confirm the in-gel kinase assay, the edg-1 expression vector and the hemagglutinin epitope (HA)-tagged ERK-2 cDNA were co-transfected into HEK293 cells, and MAP kinase activity of the HA immunoprecipitates was assayed. As shown in Fig. 6, edg-1 transfection activated the MAP kinase activity. In addition, the edg-1-induced activation of the HA-ERK-2 was suppressed by pertussis toxin. These data indicate that edg-1 signals via the Gi pathway to induce the ERK-2/MAP kinase activity.
determination of the function of orphan receptors has been onerous due to the intrinsic difficulty in the identification of ligands. For example, the mas oncogene, which was isolated due to its transforming capacity, is still uncharacterized with respect to ligands and signaling properties (43). Due to the advent of homology cloning by PCR, many orphan receptor sequences have become available. However, ligands for only a few of these orphan receptors have been found to date, for example, the cannabinoid and orphanin receptors (42, 44). In this report, we present a novel approach to define the signaling properties of the orphan receptor edg-1.

Structure-function analysis of GPRs has defined several features of the GPRs that are essential for specificity in signaling (17). While the structure of the i3 domain plays a major role in determining the specificity of G protein coupling, other intracellular loops (e.g., the C-terminal portion of intercellular loop 2 and the C-terminal tail) also contribute to the specificity (30, 31). For example, the intercellular loop 2, i3, and C-terminal domains of rhodopsin are able to interact with transducin independently (32). The i3 domain of edg-1 is only 34 residues in length and contains the Gβ-activator motif (BBXXF) as well as other potential regulatory sites (45). We expressed the i3 domain of edg-1 as a C-terminal GST fusion protein and used it as an affinity matrix to characterize G proteins that interact with it. Toxin labeling studies clearly indicate that the pertussis toxin substrates (Gαs and Gαi) but not the cholera toxin substrates (Gβγ) are capable of associating with the edg-1-i3. While we have not ruled out association of other G proteins (e.g., Gαq, Gαi2, and Gα12) with i3, our data clearly show that the Gαs and Gαi2 family of proteins bind to edg-1-i3. The interaction between Gαs and edg-1-i3 in this in vitro binding assay is highly specific, since (i) the Gαs subunits are unable to interact with GST and (ii) the interaction was suppressed by GTPγS. In addition, these data also suggested that the i3 domain alone in the C-terminal context of the soluble GST fusion polypeptide is of sufficient affinity and specificity to physically associate with the G protein α subunits.

While the in vitro association experiments proved that the GST-i3 and the Gαs associates specifically, they do not, however, demonstrate direct interaction. Thus, the yeast two-hybrid system was used to demonstrate direct physical interaction between the edg-1-i3 domain and the Gα12 polypeptide. The i3 domain was expressed as a C-terminal fusion protein with the DNA binding domain of GAL4 (GAL4-i3) and the N-terminal deleted form of the rat Gα12 polypeptide was expressed as a C-terminal fusion with the transactivation domain of the GAL4 protein (Act D-G). Interaction of two proteins results in the transactivation of the GAL4-LacZ reporter gene in the host Y190 cells (21). While the GAL4-i3 alone and Act D-G alone did not transactivate the LacZ reporter gene, co-expression of both plasmids strongly transactivated the LacZ expression, suggesting that the edg-1-i3 and the Gαi2 polypeptides interact directly. The two-hybrid system has been used previously to demonstrate direct interaction between the G protein subunits and the downstream kinases in the yeast mating pathway (46). To our knowledge, this is the first demonstration of interaction of a signaling domain of the GPR with an α subunit of a G protein. Due to the relative ease of the readout, this system holds promise as a genetic method to delineate sequences involved in the receptor/G protein interaction.

Chimeric receptor studies have indicated that multiple cyto-
solic domains of GPRs cooperate to determine specificity in G protein coupling. For example, when the I domain of the G coupled β2-adrenergic receptor was inserted into the corresponding region of the G coupled M1 muscarinic receptor, the resulting chimeric receptor stimulated both G and G pathways (47). Thus, the I domain alone is not sufficient to switch the G protein-coupling characteristics. Further chimeric studies have shown that replacement of all the cytosolic loops is sufficient to convert the G protein-coupling specificity (30–32). While the I domain of edg-1 binds to G and G, it is important to establish which G proteins are capable of coupling to intact edg-1 GPR. Thus, the epitope-tagged full-length edg-1 polypeptide was co-expressed with the individual G, G, and G polypeptides in HEK293 cells. In this system, the edg-1 polypeptide associated with G, G, and G. The association of G and G, with full-length edg-1 was not observed in transiently transfected HEK293 cells. Thus, multiple domains in the cytosolic loops of edg-1 are likely to be involved in determining the specificity in coupling to G, G, and G polypeptides. These data demonstrate that edg-1 is a Gi-linked receptor.

Recent data have supported the two-state model of receptor function; in this model receptors are in equilibrium between active (R*) and inactive (R) conformations (33). Thus, overexpression of GPRs should increase the concentration of the R* state and thereby lead to constitutive signaling (34, 35). Recent studies from in vitro transient transfection studies with orphan receptors (48) and in vivo transgenic studies with the adrenergic receptors (34, 35) support this model. Since edg-1 is an inducible receptor, regulation of signaling pathways by modulation of receptor numbers may be of physiological significance. Our data suggest that the fraction of the overexpressed edg-1 receptors that are in the activated (R*) state are capable of coupling to the G and G polypeptides for productive signaling. The Gi family of polypeptides are known to activate a number of cellular signal transduction pathways such as inhibition of the adenylyl cyclase, activation of phospholipase A2 (37, 49), induction of MAP kinase activity (36) and the activation of ion channels (50). We thus examined the activity of basal and forskolin-induced cAMP levels and the basal release of [3H]arachidonic acid release (phospholipase A2 activity) from HEK293 cells transfected with edg-1. Both the cAMP and phospholipase activities were not altered by overexpression of edg-1 with and without the G and G polypeptides (data not shown). In contrast, cellular MAP kinase activity was strongly induced by edg-1 transfection and is attenuated by pertussis toxin. It is unclear why the cellular cAMP levels and phospholipase activities are unaltered by these treatments in HEK293 cells. It is possible that edg-1 regulates the MAP kinase activity in a constitutive manner and that regulation of other pathways require the presence of the ligand(s). Alternatively, appropriate effector isoenzymes that respond to the edg-1 signal may not be present in HEK293 cells. Nevertheless, these data do confirm that edg-1-dependent MAP kinase activation occurs via the G pathway in HEK293 cells. Interestingly, the MAP kinase activity is induced in a sustained manner by edg-1 transfection. In PC-12 phaeochromocytoma cells, sustained MAP kinase activation is essential for neurite extension and differentiation (51, 52). Because edg-1 expression is associated with differentiation of endothelial cells in vitro (8), these data are consistent with a functional role for edg-1 in cellular differentiation.

In stably transfected NIH3T3 cells, edg-1 overexpression is associated with MAP kinase activation. In contrast to HEK293 cells, the edg-1 expressing NIH3T3 cells also exhibited enhanced phospholipase A2 activity. Because the cellular phospholipase A2 enzyme is phosphorylated and activated by MAP kinase (37), these data suggest that edg-1 signaling via the MAP kinase is involved in the induction of phospholipase A2 activity in NIH3T3 cells.

The MAP kinase pathway is a widely used signaling system that regulates cell growth, differentiation and apoptosis (51–57). Both GPRs as well as tyrosine kinase receptors induce the MAP kinase activity in a number of cell types (36, 49, 53–59). In fibroblasts and neuronal cells, MAP kinase activation is known to induce cell cycle traverse and differentiation, respectively (51–57, 59). For example, lysophosphatidic acid and thrombin are known to induce fibroblast proliferation via G coupled receptors (36, 60, 61). In PC-12 cells, the activation of the MAP kinase pathway regulates differentiation by inducing neurite outgrowth (51, 52). Recent studies in early Xenopus development have indicated that MAP kinase activation by the FGF receptor can account completely for the mesoderm-inducing capacity of the FGF polypeptides (12). These studies highlight the importance of the MAP kinase pathway in the control of cell growth and differentiation. The edg-1 polypeptide was originally isolated because it was induced during the in vitro differentiation and growth arrest of endothelial cells (8). While the physiological function of edg-1 is unknown at present, data presented in this report indicate that overexpression of edg-1 results in constitutive activation of the MAP kinase pathway by the Gi pathway. Whether such a mechanism plays a functional role in endothelial cell differentiation awaits further experimentation.

In conclusion, our data indicate that (i) the edg-1-I domain associates with G, G, and G polypeptides, (ii) the edg-1 receptor associates with G and G, and (iii) overexpression of edg-1 induces MAP kinase activity in HEK293 and NIH3T3 cells and (iv) edg-1 overexpression is associated with enhanced phospholipase A2 activity in NIH3T3 cells. These data provide a basis for further understanding of the function of the inducible orphan receptor edg-1.

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