Endothelin is a 21-amino acid peptide with a striking diversity of important biological responses, including vasoconstriction, bronchoconstriction, and mitogenesis. Endothelin-1 binding to the endothelin B receptor (ETB), a member of the superfamily of G-protein-coupled receptors, was associated with catalytic activation of the extracellular-regulated kinase 2 (ERK2) and stimulation of AP-1 transcriptional reporter activity. A panel of single point mutations in transmembrane helix 6 (TM6), intracellular loop 3, and transmembrane helix 7 (TM7) were developed to study the structural requirements for ETB activation. Point mutations within highly conserved regions of TM6 and intracellular loop 3 were without effect on agonist-stimulated ERK activation. However, mutations within TM7 of the ETB significantly impacted ligand-stimulated downstream signaling. For example, nine point mutations within TM7 of the ETB were identified that prevented endothelin-stimulated ERK activation. Interestingly, the TM7 mutants fell into two classes; several exhibited greatly decreased AP-1 activity, relative to wild type ETB, whereas others displayed augmented endothelin-stimulated AP-1 transcriptional activity relative to wild type ETB. Our results suggest that TM7 of the ETB is involved in its activation mechanism and regulates agonist-stimulated ERK activation.
stimulated ERK activation and had dual effects on AP-1 transcriptional activation.

**MATERIALS AND METHODS**

**Cell Culture and Transfection**

COS cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). For kinase assays cells approximately 50% confluent in 100-mm plates were transfected with the indicated plasmids (5 \( \mu \)g DNA) using LipofectAMINE (Life Technologies, Inc.). Cells were exposed to the LipofectAMINE/DNA mixture for 6 h in DMEM, the medium was changed to DMEM/10% FBS, and the cells were incubated for 48 h. Cells were serum starved by changing the medium to DMEM/0.1% FBS for 16 h, and the cells were treated with endothelin and harvested for immunoprecipitation. For immunofluorescence COS cells were grown and transfected in 0.2-μm glass slides. For AP-1 reporter assays the AP-1/luciferase plasmid was co-transfected with wild type and mutant ETB plasmids and pCMV-β-gal, which encodes β-galactosidase at a ratio of 5:1.

**Plasmid Construction**

ERK2 containing a 6-fold repeated Myc epitope fused in frame to the N-terminus was used as described previously (17). Wild type rat endothelin B receptor (GenBank accession number X57765) was subcloned into pCDNA3 containing a FLAG epitope fuse in frame at the 3’ end as described previously (17). Single point mutations were made in TM6, TM7, and the third intracellular loop of the rat ETB using the Transformer site-directed mutagenesis kit (CLONTECH) following the manufacturer’s instructions. All mutations were verified by sequencing. The AP-1/luciferase plasmid contains the AP-1 transcriptional promoter element cloned upstream of the luciferase gene (kindly provided by Dr. Mercedes Rincon, University of Vermont).

**Mutagenesis**

Single point mutations were introduced into the wild type rat ETB using the Transformer site-directed mutagenesis kit following the manufacturer’s instructions (CLONTECH). Briefly, a selection primer was engineered that changed a unique Smal site in the pCDNA3 vector at nucleotide 2093 to a XhoI site that was also unique. Additional primers were designed that changed the desired amino acids in the ETB. After synthesizing the mutant strand in situ the plasmids were transformed into a mutS Escherichia coli strain. Parental plasmids were eliminated by incubating the 125I-endothelin-1 in the presence of excess (100 nM) unlabeled endothelin. Nonspecific binding averaged 15–25% of total binding. Single point mutations were introduced into the wild type rat ETB using the Transformer site-directed mutagenesis kit following the manufacturer’s instructions (CLONTECH). Briefly, a selection primer was engineered that changed a unique Smal site in the pCDNA3 vector at nucleotide 2093 to a XhoI site that was also unique. Additional primers were designed that changed the desired amino acids in the ETB. After synthesizing the mutant strand in situ the plasmids were transformed into a mutS Escherichia coli strain. Parental plasmids were eliminated by incubating the 125I-endothelin-1 in the presence of excess (100 nM) unlabeled endothelin. Nonspecific binding averaged 15–25% of total binding.

**Luciferase Reporter Assay**

COS cells were grown in 6-well plates in DMEM containing 10% FBS. At 50% confluence, cells were transfected with 0.5 μg of ETB DNA, 0.25 μg of AP-1/luciferase reporter plasmid, and 0.1 μg of β-gal using LipofectAMINE in serum-free DMEM. After 5 h the medium was changed to growth medium, and the cells were grown for 24 h. Cells were changed to 0.1% FBS serum, incubated overnight, and then stimulated with endothelin for 6 h. The receptors were transfected into three individual culture dishes, and the values are the means ± S.D. of the three independent measurements. Cell lysates were made, and luciferase activity was measured in a scintillation counter according to the manufacturer’s instructions. All mutations were verified by sequencing. The AP-1/luciferase plasmid contains the AP-1 transcriptional promoter element cloned upstream of the luciferase gene (kindly provided by Dr. Mercedes Rincon, University of Vermont).

**Immunofluorescence**

Cells were grown in 2-well Nunc chambered slides until approximately 50% confluent and transfected with 1 μg of ETB DNA and 3 μl of LipofectAMINE, and the medium was changed to 10% FBS 5 h later. Cells were rinsed twice with phosphate-buffered saline, fixed with 4% paraformaldehyde, and permeabilized with MeOH. Nonspecific binding was blocked with 5% normal goat serum, and the cells were incubated for 1 h at room temperature in the anti-FLAG antibody (M2, Kodak Corp. New Haven, CT) at 2 μg/ml. The cells were rinsed and blocked again for 30 min in 5% normal goat serum. Cells were washed five times over 30 min in TBS-T (Tris-buffered saline with 0.1% Tween 20), incubated for 45 min in the dark at room temperature in a CY3-conjugated, affinity purified, goat anti-mouse secondary antibody (1:2000, Jackson ImmunoResearch), and mounted with VECTASHIELD (Vector laboratories Inc., Burlingame, CA) containing DAPI.
and lysed in 0.1 N NaOH. Lysates were collected, and 125I-ET content determined by liquid scintillation counting using a Packard B5005 counter.

RESULTS

Because direct structural information is not available for GPCRs, models regarding the activation mechanism of this family of receptors have relied primarily on mutagenesis and biophysical studies. Secondary structure models have provided insight into the topology of rhodopsin, including the packing of the transmembrane helices (21, 22). Recent work on rhodopsin indicates that the activation mechanism involves rigid body movement of TM6 (23). The primary sequence of the GPCR superfamily reveals several highly conserved amino acids in this helix that are also present in the ETB (Fig. 1). We wished to determine whether the activation mechanism of ETB involves TM6. Therefore, we made substitutions of several highly conserved residues in this region.

TM6 Mutants of the ETB—Our results indicate that although several residues within TM6 are almost invariably conserved among the GPCR superfamily, mutation of these residues in the ETB appeared to be without effect on ERK mitogen-activated protein kinase activation (Fig. 2). ETB mutants F331A, C334A, W335A, P337A, S341K, and K345A were co-transfected into COS cells with MT-ERK2. Endothelin stimulated ERK2 catalytic activation in the wild type ETB and mutant receptors at approximately similar levels. The basal level of ERK activity for unstimulated mutant receptors was also unchanged relative to wild type receptor (Fig. 2B). Because TM6 of the ETB is not sensitive to structural changes introduced by mutagenesis, the data infer that this helix is not integrally involved in the activation mechanism of this receptor.

Intracellular Loop 3 Mutants of the ETB—Several studies have implicated the third intracellular loop of GPCRs, such as the adrenergic and muscarinic receptors, as playing a role in downstream effector function (20, 25). Therefore, we also examined the third loop of the ETB by introducing amino acid substitutions into this area and expressing the mutant receptors in COS cells with MT-ERK2. The third loop of the ETB appeared to be relatively unaffected by mutation. ETB mutants M299A, T323A, M306A, and D312A had essentially wild type levels of ERK activation, whereas ETB mutants Q316A and K322A were slightly reduced in their ability to activate ERK (Fig. 3). As with the TM6 region of the ETB, analysis of the basal activity of ERK in cells transfected with mutant receptors revealed levels of activity comparable with those of unstimulated wild type receptors (Fig. 2B).

TM7 Mutants of the ETB—Transmembrane helix 7 of the ETB shares relatively little homology with other GPCRs, and in fact the GPCR superfamily has little overall homology in this region (Fig. 1). However, TM7 of the ETB was very sensitive to mutation. A panel of TM7 mutants were engineered and co-transfected into COS cells with MT-ERK2. ETB mutants M373K, N377P, S378K, I380A, P382K, L385A, V388K, and K390A were...
all severely compromised in their ability to activate ERK, in comparison with wild type (Fig. 4). Western blotting of the COS cell lysates demonstrated that MT-ERK2 was expressed at similar levels across the experiment, confirming that the decrease in catalytic activity was due to decreased activation of MT-ERK2 and not lower expression of the kinase. Although several TM7 mutant receptors revealed slightly elevated levels of basal ERK activity relative to wild type receptor (Fig. 2B), this was not reflected in an increase in ET-stimulated ERK activity relative to wild type receptors. This suggests that introduced mutations did not produce catalytically active mutants.

**FIG. 3.** Loop 3 of the ETB is not sensitive to mutation. Amino acid substitutions were introduced into the intracellular loop 3 region of the ETB and expressed in COS cells as in Fig. 2. The mutant receptors were activated by ligand binding, and ERK kinase activity was measured by immune complex kinase assay using MBP as an *in vitro* substrate. Basal activity of Loop 3 mutants is presented in Fig. 2B. The results indicate that the mutant receptors stimulate ERK in response to ET to a similar degree as do the wild type receptors (ETB-WT), suggesting that loop 3 is not sensitive to structural changes with respect to ERK activation.

**FIG. 4.** TM7 regulates receptor-mediated activation of ERK. Amino acid substitutions were introduced into the TM7 region of the ETB, and the wild type (ETB-WT) and mutant receptors were co-transfected with MT-ERK2 into COS cells. Kinase assays were done using MBP as an *in vitro* substrate to measure catalytic activity. Basal activity is presented in Fig. 2B. Several of the point mutations significantly decreased endothelin-stimulated ERK activation. Western blots were done to document that decreases in ERK kinase activity reflected changes in the activation of the enzyme rather than its expression level.
ETB Mutant Receptors Are Expressed and Properly Localized—Introducing nonconserved amino acid changes in the ETB could reduce its downstream signaling capabilities as a result of a gross alteration in the topology of the receptor. Therefore, we investigated the expression levels and localization of the ETB mutant receptors by immunofluorescence. Receptors bearing a FLAG tag on their carboxyl terminus were expressed in COS cells and processed for immunofluorescence using the anti-FLAG monoclonal antibody (Fig. 5). The mutant receptors were expressed and localized in essentially the same manner as the wild type receptor. The data indicate that the inability of the TM7 mutations to activate ERK resulted from a defect in the activation mechanism, rather than a mutation-induced effect on expression or localization of the mutant receptors.

TM7 of the ETB Regulates AP-1 Transcriptional Activation—Ligand binding to the ETB triggers a diverse spectrum of biological responses, depending on the target tissue in which the receptor is expressed. ETBs stimulate mitogenesis in selective cell types, and this response is thought to involve the activation of key transcriptional events, including AP-1 activity (14). Furthermore, catalytically activated ERK is known to phosphorylate transcription factors involved in AP-1 activity (26) and stimulate transcription. Therefore, we wished to determine whether ERK activation is required for AP-1 transcriptional activation. To this end we have examined transcriptional activation of an AP-1 luciferase reporter construct by wild type and mutant ETBs (8). The mutants fell into two distinct classes based on their ability to stimulate AP-1 transcriptional activation. Several of the mutations in TM7 of the ETB significantly decreased endothelin-stimulated AP-1 transcriptional activation. For example, N377P, S378K, P382K, A384K, L385A, K390A, and F392A were compromised in their ability to stimulate AP-1 reporter activity when exposed to ligand. Interestingly, a second class of TM7 mutant was identified that displayed augmented endothelin-stimulated AP-1 transcriptional activity. TM7 mutants D367A, N372A, I383A, V388K, and I380A when stimulated with ligand demonstrated augmented AP-1 activity compared with wild type ETB (Fig. 6). The I380A TM7 mutant had the largest increase in ligand-stimulated AP-1 activity. These mutants were consistently observed to have increased endothelin-stimulated AP-1 activity; however, the basal AP-1 activity was essentially the same as wild type (data not shown). The results suggest that TM7 of the ETB is integrally involved in ligand-induced activation of AP-1 activity because mutations were identified that both prevented and augmented AP-1 responses.

Ligand Binding Properties of the Mutant ETBs—The ternary complex model defines the dynamic interactions between receptor-G-protein interactions and ligand binding (18). High
affinity agonist binding is thought to be dependent on efficient G-protein interactions with the receptor. Furthermore, several constitutively activated GPCRs are found to have increased affinity for agonist (19, 20, 25). The ternary complex model suggests that receptor mutations that decrease or interfere with proper receptor-G-protein physical interactions will result in altered agonist binding.

We conducted binding studies on both purified membranes and transfected, whole cell preparations for each receptor mutant. Our results are consistent with this model. Several of the TM7 mutants displayed a range of \(^{125}\)I-endothelin binding, which was generally lower than the wild type receptor, suggesting that the mutant receptors were impaired in their ability to couple with G-protein (Table I and Fig. 7). Other mutants displayed binding closer to wild type levels but were unable to efficiently activate ERK or stimulate AP-1 luciferase activity (N377P and S378K mutants; Figs. 6 and 7 and Table I). Inefficient receptor-G-protein interactions may therefore account for the inability of ETB mutants to activate ERK. Furthermore, any decreased binding was not a result of a lack of receptor expression or localization, because the mutant ETBs were expressed and localized in a manner similar to that of the wild type receptor (Fig. 5).

**DISCUSSION**

GPCRs form an important and diverse group of cell surface receptor proteins that have yet to be crystallized for structural studies. Mutagenesis experiments involving the major classes of GPCRs have provided a comprehensive picture regarding the molecular details of receptor-ligand interactions and receptor-G-proteins interactions. Studies with rhodopsin suggest a model of activation that involves interaction of TM3 and TM6. In the case of rhodopsin, rigid body movement of TM6 is thought to be induced by agonist binding and to alter the conformation of the binding site for transducin, the associated heterotrimeric G-protein (23).

In our experiments we have introduced a series of point mutations in TM6, intracellular loop 3, and TM7 to define the regions of the ETB that are involved in downstream signaling. Our results provide insight into the activation mechanism of the ETB. The TM6 domain of the ETB has several residues that are highly conserved across a broad spectrum of GPCRs, and studies with rhodopsin implicate this region in the ligand-induced activation mechanism. Therefore, we postulated that these residues may be important in regulating the coupling of the ETB with downstream effectors, such as ERK activation. Surprisingly, systematic substitution of the highly conserved residues of TM6 had no effect on activation of ERK (Fig. 2). Although these residues are highly conserved, this area of the ETB does not appear to be sensitive to structural changes. The
results argue against TM6 as being critically involved in ligand-induced ERK activation. The ETB appears to be distinct from at least two other GPCRs in which TM6 is involved in the activation mechanism. For example, in the case of the m5 muscarinic receptor, substitution of Ser465 at the extracellular junction of TM6 resulted in activation of the receptor (20). Substitution of Ala293 of the a1B-adrenergic receptor, which is in loop 3 close to the junction of TM6, resulted in constitutive activation of the receptor (25). Amino acid residues throughout TM6 and loop 3 of the ETB were mutated without effect on basal or ligand-induced ERK activation. Although these regions in the muscarinic and adrenergic receptors are involved in the activation mechanism of the receptors, this theme is not applicable to the ETB.

The intracellular loops of GPCRs form a structure in the cytosol of the cell that may be important in mediating protein-protein interactions with heterotrimeric G-proteins. In fact, introducing mutations into this area can impair downstream signaling functions (25). In the case of the ETB, the putative intracellular loops are very small, relative to other GPCRs. The intracellular loop 3 of the ETB is the largest and was therefore hypothesized as most likely to be involved in coupling to G-proteins and downstream signaling functions. We therefore systematically introduced point mutations into the ETB loop 3 area and evaluated the ability of the mutant receptors to activate the ERK pathway. Our results indicate that, similar to the TM6 region, loop 3 is not sensitive to the introduction of non-conserved amino acid substitutions. Several mutations were introduced into loop 3 of the ETB with no effect on ligand-stimulated ERK activation.

In contrast, TM7 of the ETB proved to be very sensitive to mutation. Nine residues in TM7 were identified that, when substituted with a nonconserved residue, resulted in a significant decrease in ligand-stimulated ERK activation (Fig. 4). Our results differ from the rhodopsin model of GPCR activation to the extent that TM6 is postulated to be critically involved in ligand-induced activation of rhodopsin, whereas this region of the ETB does not appear to be important for ERK activation. In contrast, TM7 of the ETB was very sensitive to mutation, with respect to agonist-induced ERK activation. In the three-dimensional model of helix packing of rhodopsin, helix 7 is close to helix 6. Because helix 6 is thought to move during activation, it is possible that mutations of helix 7 sterically interfere with the ability of helix 6 to be displaced during ligand binding. However, if helix 6 of the ETB were critically involved in activation, a reasonable prediction would be that it should be sensitive to mutagenesis. Therefore, the data suggest the possibilities that the activation mechanism of the ETB is distinct from that of rhodopsin and that helix 7 of the ETB is involved in ligand-induced activation.

Some of the biological effects of ETB activation are thought to involve activation of AP-1 mediated transcription (14).
cause ERK is known to phosphorylate transcription factors leading to increased AP-1 activity (26), we examined the relationship between ETB-stimulated ERK activation and AP-1 transcriptional activation. Seven of the residues in TM7 that were sensitive to mutation, with respect to ERK activation, demonstrated reduced AP-1/luciferase reporter activity, suggesting that AP-1 activation and ERK activity are positively correlated. Interestingly, there were five notable exceptions to this correlation. ETB TM7 mutants D367A, N372A, I383A, V388K, and I380A consistently demonstrated increased endothelin-stimulated AP-1 activity. The I380A TM7 mutant was especially notable because it consistently demonstrated augmented ligand-stimulated AP-1 activation. The basal ERK activity of the receptor mutants was similar to wild type levels with minor elevations in some TM7 mutants (F392A, S378K, p382K, and K390A). However, no positive correlation could be shown between increased basal ERK activity and increased AP-1 transcription (compare Fig. 2B with Fig. 6). With the exception of D367A, the mutants that had increased endothelin-stimulated AP-1 activity were unable to stimulate ERK kinase activity (Figs. 4 and 6). The results suggest that ERK activation and AP-1 activation are separable, and that ERK activity is not an absolute requirement for AP-1 activation. The mechanism whereby AP-1 is activated may involve one or more of the several signal transduction systems known to be activated by endothelin. Additional studies will be required to dissect the layers of interaction between the components of the endothelin signaling pathway.

Acknowledgment—We thank Dr. Mercedes Rincon for providing plasmids.

REFERENCES
1. Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. & Masaki, T. (1988) Nature 332, 411–415
2. Whelchel, A., Evans, J. & Posada, J. (1997) Am. J. Respir. Cell Mol. Biol. 16, 589–596
3. Lin, H. Y., Kaji, E. H., Winkel, G. K., Ives, H. E. & Lodish, H. F. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3185–3189
4. Arai, H., Horii, S., Aramori, I., Ohkubo, H. & Nakanishi, S. (1990) Nature 348, 730–732
5. Sakurai, T., Yanagisawa, M., Takauwa, Y., Miyazaki, H., Kimura, S., Goto, K. & Masaki, T. (1990) Nature 348, 732–735
6. Shapiro, P. S., Evans, J. N., Davis, R. J. & Posada, J. A. (1996) J. Biol. Chem. 271, 5750–5754
7. Bogoyevitch, M. A., Marshall, C. J. & Sugden, P. H. (1995) J. Biol. Chem. 270, 26303–26310
8. Rincon, M. & Flavell, R. A. (1996) Mol. Cell. Biol. 16, 1074–1084
9. Simonson, M. S. & Herman, W. H. (1993) J. Biol. Chem. 268, 9347–9357
10. Simonson, M. S., Wang, Y. & Herman, W. H. (1996) J. Biol. Chem. 271, 77–82
11. Foschi, M., Chari, S., Dunn, M. J. & Sorokin, A. (1997) EMBO J. 16, 6439–6451
12. Cazaubon, S. M., Ramirez-Morales, F., Fischer, S., Schweighoffer, F. Strosberg, A. D. & Couraud, P-O. (1994) J. Biol. Chem. 269, 24805–24809
13. Daub, H., Weiss, F. U., Wallasch, C. & Ulrich, A. (1996) Nature 379, 557–560
14. Simonson, M. S., Jones, J. M. & Dunn, M. J. (1992) J. Biol. Chem. 267, 8643–8649
15. Hashido, K., Adachi, M., Gamou, T., Watanabe, T., Furuchi, Y. & Miyamoto, C. (1993) Cell. Mol. Biol. Res. 39, 3–12
16. Adachi, M., Hashido, K., Trzeciak, A., Watanabe, T., Furuchi, Y. & Miyamoto, C. (1993) J. Cardiovasc. Pharmacol. 22, Suppl. 8, 121–124
17. Aguilla, E., Whelchel, A., Knott, H. J., Nelson, M. & Posada, J. (1996) J. Biol. Chem. 271, 31572–31579
18. De Lean, A., Stadel, J. M. & Lefkowitz, R. (1980) J. Biol. Chem. 255, 7108–7117
19. Samama, P., Cotecchia, S., Costa, T. & Lefkowitz, R. J. (1993) J. Biol. Chem. 268, 4626–4636
20. Spalding, T. A., Burstein, E. S., Wells, J. W. & Brann, M. R. (1997) Biochemistry 36, 10109–10116
21. Dratz, E. A. & Harbarve, P. A. (1983) Trends Biochem. Sci. 8, 128–131
22. Unger, V. M. & Schertler (1995) Biophys. J. 68, 1776–1786
23. Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L. & Khorana, H. G. (1996) Science 274, 768–770
24. Franke, R. R., Koenig, B., Sakmar, T. P., Khorana, H. G. & Hoffman, K. P. (1990) Science 250, 123–125
25. Kjelsberg, M. A., Cotecchia, S., Ostrowski, J., Caron, M. G. & Lefkowitz, R. J. (1992) J. Biol. Chem. 267, 1439–1443
26. Karin, M. (1995) J. Biol. Chem. 270, 16483–16486