Mutation of Peptide Binding Site in Transmembrane Region of a G Protein-coupled Receptor Accounts for Endothelin Receptor Subtype Selectivity*

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Stanley R. Krystek, Jr.1, Pramathesh S. Patel,†, Patricia M. Rose‡, Susan M. Fishers,§, Bernadette K. Kienzle,¶, David A. Lach,‖, Eddie C.-K. Lui,§ Jean S. Lynch,¶ Jiri Novotny,‖ and Maria L. Webb∗∗

From the Departments of 1Macromolecular Modeling, 2Microbial Molecular Biology, 3Metabolic Diseases, and 4Cardiovascular Biochemistry, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543

The molecular basis for endothelin (ET) isopeptide selectivity between ETα and ETβ receptors was studied by examining ligand binding to several site-specific mutants of the human ETα receptor. Based on a computer-built three-dimensional model of the ETα receptor, five non-conserved amino acids, clustered around the putative ligand binding site, were targeted for mutation to alanine. Expression of the wild-type and mutant ETα receptors in COS-7 cells revealed that the binding profile of one of the ETα mutants, Tyr129→Ala, was characteristic of the ETβ receptor. In the Tyr129→Ala ETα receptor mutant the affinity of two ETα-selective agonists, endothelin-3 and sarafotoxin S6c, was increased 10-200-fold, whereas that for two ETβ-selective antagonists, BQ-123 and BMS-182874, was reduced 350-2,000-fold. Thus, mutation of a single amino acid in the second transmembrane region of the wild-type ETα receptor results in subtype conversion. In addition, these data represent the first example of peptide interactions with a transmembrane region of a G protein-coupled receptor and indicate that Tyr129, located in the second transmembrane region of the ETα receptor, is a critical component for determination of endothelin receptor subtype-selective ligand binding.

The endothelin (ET) and sarafotoxin peptides comprise a family of structurally and pharmacologically related agents with potent biological activity (1). Originally isolated from porcine endothelial cells (2), endothelin has now been shown to be produced by numerous tissues including lung, kidney, eye, gastrointestinal tract, and many nuclei in the central nervous system (3, 4). Among the actions exerted by endothelins are vasconstriction and vasodilation of smooth muscle (4-6), pressor and depressor (2, 7) effects, positive myocardial inotropy and chronotropy (8), and mitogenicity (9). These diverse actions are widely attributed to the existence of multiple endothelin receptor subtypes with discrete and regulated cellular distributions and functions.

Due largely to the potent and long acting vasoconstrictor effects of the endothelin isopeptides on vascular and non-vascular smooth muscle, the endothelin receptors have been proposed as targets for therapeutic intervention in numerous diseases (10, 11). Two receptor subtypes for mammalian endothelins have been identified on the basis of molecular (12, 13) and pharmacological (1, 14) evidence. Comparison of deduced amino acid sequences for the ETα and ETβ subtypes reveals that these proteins are 59% identical and are members of the putative heptahelical receptor family that is G protein-coupled. The level of conservation is greater in the intracellular loops and transmembrane regions where the sequence identity is 75%. Despite this level of homology between subtypes, ETα and ETβ receptors are distinguished by differential affinities for peptidic and nonpeptidic ligands. The ETα receptor subtype binds ET-3 and sarafotoxin S6c with low affinity and BQ-123 and BMS-182874 with high affinity. Conversely, the ETβ receptor subtype binds ET-3 and sarafotoxin S6c with high affinity and BQ-123 and BMS-182874 with low affinity.

Recent reports describing chimeric ETα and ETβ receptors have attempted to address the molecular basis of subtype-selective ligand binding. These studies demonstrated that transmembrane regions 1, 2, 3, and 7 (15), as well as the first extracellular loop (16), compose a subdomain for the ETα antagonist, BQ-123, whereas critical binding determinants for ETβ-selective agonists appear to reside in transmembrane regions 4-6 (15). However, the precise amino acid residues involved in the binding site for peptidic or nonpeptidic ligands remain to be identified. Elucidation of the structural determinants involved in ligand binding is critical to understanding the mechanism of ET receptor-ligand interactions and to rational drug development. To this end, we developed a computer-generated three-dimensional model of the ETα receptor to guide site-directed mutagenesis aimed at probing ligand-selective interactions with this receptor. Here we report that Tyr129 in the second helix of the ETα receptor is a critical determinant of subtype A-selective ligand binding.

MATERIALS AND METHODS

Model Building—Sequences of the human ETα and ETβ receptor subtypes were aligned, and pairwise comparisons of amino acid hydrophobicity values were made using the Goldman, Engelman, Steitz hydropathy scale (17), which produced the conservation value as: hydropathicity conservation value = scale range (H - H2), where the scale ranges from most hydrophobic (F = 3.7) to most hydrophilic (R = 12.3) and H and H2 are values for the corresponding ETα and ETβ amino acid pair being compared (18). The identified transmembrane sequences were then threaded through the seven α-helices of bacteriorhodopsin (19). Because proline residues introduce kinks into straight helices with an average kink angle, θ, of 28° (±5°) (20) and helix kinking is important for shaping the size of the putative ligand binding cavity (20, 21), helical segments in bacteriorhodopsin containing kinks caused by prolines were brought to standard α-helical φ, ψ values (57-74°) by constrained minimization. Helices in the ETα model containing prolines were then kinked by constrained minimization of selected torsional angles until the kink angle θ was within average values (see above). Molecular modeling was conducted with INSIGHT and DISCOVER ( Biosym Technologies, San Diego, CA) and GRASP (A. Nicholls, Colum-

1 The abbreviation used is: ET, endothelin.
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Bia University) with interactive graphics display and calculations conducted on a Silicon Graphics 4D/440 work station. The geometry of the seven-helix bundle was further optimized using molecular mechanics calculations as implemented in DISCOVER, followed by placement of side chains using the conformational search procedure CONGEN (22).

Cloning and Mutagenesis of Mutant Receptors—Site-directed mutagenesis of the human placental ET<sub>1</sub> receptor cDNA (23) was conducted using the method described by Deng and Nickoloff (24). Briefly, the wild-type ET<sub>1</sub> receptor cDNA was subcloned into a truncated vector, pACYC184 designed to maximize the number of unique restriction sites. Oligonucleotide(s) that introduced the desired mutation, created an analytical restriction site, and destroyed a unique restriction site were synthesized and purified (Applied Biosystems 391A synthesizer, Palo Alto, CA). The modified DNA was used to transform mutS Escherichia coli, and plasmid DNA was prepared and digested with the appropriate restriction enzyme. The resulting digestion linearized the unmutated DNA while leaving the mutated DNA circular. Transformation of K-12 E. coli yielded colonies, the majority of which contained plasmid DNA with the desired mutation. Mutant plasmids were identified by restriction analysis and subsequently verified by sequencing the entire cDNA clone (25). The wild-type and mutant receptor cDNAs were then subcloned into the mammalian expression vector, pcDNA3.

Expression of Receptors and Radioligand Binding—Wild-type and mutant ET<sub>1</sub> and ET<sub>2</sub> receptor cDNAs were transfected into COS-7 cells using the polycationic lipid Lipofectamine (Life Technologies Inc.) according to the manufacturer's instructions. Cells were harvested 48–72 h after transfection in buffer A (Dulbecco's modified Eagle's medium containing 20 mm Hepes pH 7.4 at 37 °C, 0.1 mm phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, Polytron-homogenized, and centrifuged at 100,000 × g for 1 h at 4 °C. The supernatant was discarded and the membrane pellet was resuspended in buffer A. Membranes were homogenized and stored in aliquots at −80 °C. Radioligand binding was conducted as previously described (26). COS-7 cell membranes (0.5–10 μg of protein) were incubated with 30–50 pmol ET<sub>1</sub> in the presence of increasing concentrations of competitor for 2 h at 37 °C. Nonspecific binding was defined in the presence of 100 nM ET-1. Data were analyzed by iterative curve fitting to a 1- or 2-binding site model, and K<sub>i</sub> values were calculated from IC<sub>50</sub> values (27).

**RESULTS AND DISCUSSION**

In previous model building of various heptahelical receptors, the seven transmembrane α-helices were typically predicted from amino acid sequence-derived hydrophobicity based on alignment to bacteriorhodopsin (28–36). In an attempt to more accurately determine helical boundaries of transmembrane regions, we have used a hydrophobicity conservation matrix (17, 18) in conjunction with sequence similarity. In a blind test, this procedure correctly identified the transmembrane regions in bacteriorhodopsin to within 1 amino acid residue. Following construction, the ET<sub>1</sub> receptor model was analyzed for amino acid residues which 1) were predicted to be in the transmembrane regions (170 total residues), 2) differed between ET<sub>1</sub> and ET<sub>2</sub> subtypes (32 residues), 3) represented non-conservative amino acid pairs (side chains colored conservative and discarded from selection were Val:ILE, Val:Leu, Met:ILE, Leu:MET, and Ala:SER) between ET<sub>1</sub> and ET<sub>2</sub> subtypes (12 residues), 4) were predicted to be at the extracellular surface (5 residues), and 5) were predicted to be in the putative binding cavity (2 residues; see Fig. 1A). These two amino acid residues, Tyr<sup>229</sup> and Ser<sup>197</sup> in the second and third transmembrane regions, respectively (see Fig. 1B), were targeted for alanine replacement by site-directed mutagenesis (23, 24). In addition, the three other amino acids (Val<sup>225</sup>, Gly<sup>261</sup>, and Phe<sup>320</sup>) predicted to be near the extracellular surface were mutated as a test of the ET<sub>1</sub> model.

Mutant and wild-type ET<sub>1</sub> receptors and wild-type ET<sub>2</sub> receptors were transiently expressed in COS-7 cells, and the af-

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**Table 1**

| Agent          | Wild-type ET<sub>1</sub> | Y129A ET<sub>1</sub> | S167A ET<sub>1</sub> | V225A ET<sub>1</sub> | G261A ET<sub>1</sub> | F320A ET<sub>1</sub> | Wild-type ET<sub>1</sub> |
|----------------|-------------------------|---------------------|---------------------|---------------------|---------------------|---------------------|-------------------------|
| ET-1           | 0.2 ± 0.07              | 0.3 ± 0.1           | 0.3 ± 0.1           | 0.2 ± 0.1           | 0.7 ± 0.1           | 0.3 ± 0.1           | 0.1 ± 0.0               |
| ET-2           | 0.2 ± 0.02              | 0.5 ± 0.1           | 2.630 ± 210         | 185 ± 30            | 1,520 ± 125         | 0.2 ± 0.1           | 0.1 ± 0.0               |
| Sarafotoxin S6c| 14,450 ± 1,950          | 1,100 ± 360         | 6,000               | 0.1 ± 0.0           | 32,800 ± 1,200      | 0.1 ± 0.0           | 32,800 ± 1,200          |
| BQ-123         | 19 ± 3                  | 41,100 ± 1,000      | 5 ± 0.7             | 3 ± 0.5             | 34,400 ± 1,000      | 0.1 ± 0.0           | 32,800 ± 1,200          |
| BMS-182874     | 170 ± 20                | 60,000 ± 4,000      | 500 ± 20            | 170 ± 40            | 190 ± 15            | 55,000 ± 13,500      | 50,000 ± 13,500         |
| Ro 46–2005     | 200 ± 20                | 270 ± 20            | 130 ± 5             | 310 ± 10            | 350 ± 70            | 0.1 ± 0.0           | 32,800 ± 1,200          |

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**FIG. 1.** Model of the helical core of the ET<sub>2</sub> receptor viewed from the extracellular surface. A, molecular surface of the receptor was generated using GRASP (A. Nicholls, Columbia University) showing the mutated residues colored in green, Tyr<sup>229</sup> and Ser<sup>197</sup>; red, Val<sup>225</sup> and Phe<sup>320</sup>; orange, Gly<sup>261</sup> Gly<sup>261</sup> being buried in the interior or transmembrane helix 5 does not contribute to the molecular surface. Closest atoms on the surface were colored instead to show the approximate location of the glycine. B, axial view of the extracellular face of the receptor model showing residues comprising the presumed ligand binding site. Transmembrane helices are numbered I through VII. Amino acids displayed in space-filling mode and colored in green are the 5 residues targeted for site-directed mutation. Other residues colored in purple are those exposed in the extracellular cavity. C, cross-sectional view of the ET<sub>1</sub> receptor model with residues colored as described for B with the extracellular surface oriented toward the top.
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The results with the Tyr^129 → Ala ET_A mutant suggest that these endothelin isopeptides and peptidic antagonist interact with Tyr^129 in the second transmembrane region of the ET_A receptor. Indeed, because the affinity of BMS-182874 is also affected by the Tyr^129 → Ala mutation, these data indicate that the naphthalene sulfonamide antagonist also interacts with Tyr^129. This represents the first example of a peptide interaction with a transmembrane region of a G protein-coupled receptor.

In summary, this is the first report describing a specific component of the ET_A receptor binding site. To the extent that all the surface-exposed, receptor subtype-variable amino acids were evaluated by alanine-scanning mutagenesis, the data reported here indicate that Tyr^129 in transmembrane region 2 is involved in determining subtype-selective peptidic and nonpeptidic (agonist and antagonist) ligand binding and contributes to fine selectivity in binding to endothelin receptors.

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REFERENCES

1. Massaki, T., Kimura, S., Yanagisawa, M., and Goto, K. (1991) Circulation 84, 1457–1468
2. Yanagisawa, M., Kuribara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Goto, K., and Massaki, T. (1988) Nature 333, 411–415
3. Yanagisawa, M., and Masaki, T. (1989) Trends Pharmacol. Sci. 10, 374–378
4. Simonson, M., and Dunn, M. (1990) J. Clin. Invest. 85, 790–797
5. Secrest, R. J., and Cohen, M. I. (1985) Life Sci. 36, 1363–1372
6. Warner, T. D., de Nucci, G., and Vane, J. R. (1989) Eur. J. Pharmacol. 169, 235–236
7. Martin, R. R., Maraden, P. A., Brenner, B. M., and Ballermann, B. J. (1989) Biochem. Biophys. Res. Commun. 162, 130–137
8. Ishikawa, T., Yanagisawa, M., Kimura, S., Goto, K., and Masaki, T. (1988) Am. J. Physiol. 255, H707–H709
9. Weber, H., Webb, M. L., Serafini, R., Taylor, D. S., Moreland, S., Norman, J., and Molloy, C. J. (1994) Mol. Endocrinol. 8, 148–158
10. Nakayama, Y. (1990) Trends Pharmacol. Sci. 11, 96–98
11. Yanagisawa, M., Inoue, A., Ishikawa, T., Kurihara, H., Kimura, S., Komatsu, T., Watanabe, T., Tsukahara, S., Goto, K., and Masaki, T. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 6964–6967
12. Araki, H., Hori, S., Aramori, I., Okkubo, H., and Nakashima, S. (1990) Nature 349, 730–732
13. Sakurai, T., Yanagisawa, M., Takahara, Y., Miyazaki, H., Kimura, S., Goto, K., and Masaki, T. (1990) Nature 349, 732–735
14. Rubanyi, G. M., and Parker Botelho, R. (1991) FASEB J. 5, 2713–2720
15. Sakurai, T., Yanagisawa, M., Sawamura, T., Enoki, T., Ohtani, T., Sakurai, T., Nakao, K., Toyooka, T., and Massaki, T. (1993) J. Biol. Chem. 268, 8547–8553
16. Asahi, M., Yang, Y.Y., Toczylowski, A., Furuchi, Y., and Miyamoto, C. (1992) FEBS Lett. 311, 179–183
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17. Engleman, D. M., Steitz, T. A., and Goldman, A. (1986) *Ann. Rev. Biophys. Biophys. Chem.* 15, 321–353
18. Riek, P., Harabagian, M., Sung, S.-S., Novotny, J., and Graham, R. M. (1981) *FASEB J.* 5, A396
19. Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckmann, E., and Downing, K. H. (1990) *J. Mol. Biol.* 213, 899–929
20. Barlow, D. J., and Thornton, J. M. (1988) *J. Mol. Biol.* 210, 601–619
21. von Heijne, G. (1991) *J. Mol. Biol.* 218, 499–503
22. Bruccoleri, R. E. (1993) *Mol. Simulation* 10, 151–174
23. Hayzer, D., Rose, P. M., Lynch, J. S., Webb, M. L., Kienzle, B. K., Liu, E. C.-K., Bogosian, E. A., Brinson, E., and Runge, M. S. (1992) *Am. J. Med. Sci.* 304, 231–238
24. Deng, W. P., and Nickoloff, J. A. (1992) *Anal. Biochem.* 200, 814–818
25. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* 74, 5463–5467
26. Webb, M. L., Liu, E.-C.K., Monshizadeh, H., Chao, C.C., Lynch, J., Fisher, S. M., and Rose, P. M. (1983) *Mol. Pharmacol.* 44, 959–965
27. Cheng, Y. C., and Prusoff, W. H. (1973) *Biochem. Pharmacol.* 22, 3099–3108
28. Findlay, J., and Eliopoulos, E. (1990) *J. Biol. Chem.* 266, 15481–15482
29. Strader, C. D., Sigal, I. S., and Dixon, R. A. F. (1989) *FASEB J.* 3, 1825–1832
30. Dehman, H. G., Thorner, J., Caron, M. G., and Lefkowitz, R. J. (1991) *Annu. Rev. Biochem.* 60, 653–688
31. Maloney-Huss, K., and Lybrand, T. P. (1992) *J. Mol. Biol.* 225, 859–871
32. Pardo, L., Ballesteros, J. A., Osman, R. O., and Weinstein, H. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 4009–4012
33. Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J., and Sealson, S. C. (1992) *DNA Cell Biol.* 11, 1–20
34. Trump-Kallmeyer, S., Hoefack, J., Brinivols, A., and Hibert, M. (1992) *J. Med. Chem.* 35, 3448–3462
35. Cronet, P., Sander, C., and Friend, G. (1993) *Protein Eng.* 6, 59–64
36. Higgins, J. P., Trump-Kallmeyer, S., Hibert, M. F., Hoffack, J. M., Fanger, B. O., and Jones, C. R. (1993) *Eur. J. Pharmacol. Mol. Pharmacol.* Sect. 245, 293–294
37. Ibats, M., Noguchi, K., Sasaki, T., Fukuroda, T., Tsuchida, S., Kimura, S., Fukuami, T., Ishikawa, K., Nishikibe, M., and Yano, M. (1992) *FASEB J.* 6, 5463–5467
38. Stein, P. D., Hunt, J. T., Floyd, D. M., Moreland, S., Dickenson, K. E. J., Mitchi, C., Liu, E. C.-K., Webb, M. L., Murugesan, N., Dickey, J. M., McClun, D., Zhang, R., Lee, V. S., Saffo, R., Delaney, C., Schaffer, T. R., and Kozloski, M. (1994) *J. Med. Chem.* 37, 329–331
39. Colozl, B., Breu, V., Burri, K., Cassal, J.-M., Fisch, W., Gray, G. A., Hirth, G., Leffler, B.-M., Muller, M., Neidhart, W., and Ramuz, H. (1993) *Nature* 365, 759–761
40. Tota, M. R., and Strader, C. D. (1990) *J. Biol. Chem.* 265, 16891–16897
41. Suryanarayana, S., Duant, D. A., Zastrow, M. V., and Kobilka, B. K. (1991) *J. Biol. Chem.* 266, 15488–15492
42. Guan, X., Peroutka, S. J., and Kobilka, B. K. (1992) *Mol. Pharmacol.* 41, 688–698
43. Oksenberg, D., Marsters, S. A., O'Dowd, B. F., Jin, H., Havlik, S., Peroutka, S. J., and Ashkenasi, A. (1992) *Nature* 360, 161–163
44. Beilborn, M., Lee, Y.-M., McBride, E. W., Quinn, S. M., and Kopin, A. S. (1993) *Nature* 362, 348–350
45. Fong, T. M., Cascieri, M. A., Yu, H., Bansal, A., Swain, C., and Strader, C. D. (1993) *Nature* 362, 250–253
46. Sachais, B. S., Snider, R. M., Lowe, J. A., III, and Krause, J. E. (1993) *J. Biol. Chem.* 268, 2319–2333
47. Wachterh, H. L., Chazan, B. D., Nagyana, Y., Russo, D., and Rappoport, B. (1990) *Science* 249, 1423–1425