Distinct Subdomains of Human Endothelin Receptors Determine Their Selectivity to Endothelin\textsubscript{A}-selective Antagonist and Endothelin\textsubscript{B}-selective Agonists*  

The endothelin (ET) family of peptides acts via two subtypes of G-protein-coupled heptahelical receptors termed ETA and ETB, which have distinct rank orders of affinity to endothelin receptor agonists and antagonists. To delineate which portions of the receptor molecules determine ligand selectivity, we have constructed a series of chimeras between human ETA and ETB receptors and characterized the chimeric receptors expressed in heterologous cell lines by competitive radioligand binding analysis and by measuring agonist-induced transients of intracellular Ca\textsuperscript{2+}. We demonstrate that the binding determinant for the ETA-selective agonists ET-3, BQ3020, and IRL1620 resides within the region spanning the putative transmembrane helices IV-VI and the adjacent loop regions. In contrast, the transmembrane helices I, II, III, and VII plus the intervening loop regions specify the selectivity for BQ123, an ET\textsubscript{A}-selective antagonist. BQ123 exhibited no detectable agonistic activity in all wild-type and chimeric receptors tested. A chimeric receptor that has the transmembrane helices IV-VI (and adjacent loops) from the ETA receptor binds both the ETA- and ETB-selective ligands with high affinities. Moreover, BQ123 competitively inhibits the binding of the amino-terminally truncated ET\textsubscript{B} agonists, \textsuperscript{125}I-BQ3020 and \textsuperscript{125}I-IRL1620, to this chimeric receptor, suggesting that BQ123 is a mimic of the carboxy-terminal linear portion of endothelins. These findings indicate that there are at least two separable ligand interaction subdomains within the endothelin receptors.

The endothelins are a family of potent vasoactive peptides termed endothelin-1, -2 and -3 (ET-1, -2 and -3)\textsuperscript{1} (1, 2). The first member of the family, ET-1, was initially described as a potent vasoconstrictor produced by vascular endothelial cells (3). They all consist of 21 amino acid residues with two intramolecule disulfide bonds formed between Cys\textsuperscript{1}-Cys\textsuperscript{11} and Cys\textsuperscript{20}-Cys\textsuperscript{21}. The amino acid sequence of each member of the family exhibits a nearly perfect conservation among mammalian species; the only species-related sequence difference known to date is the substitution of Ser\textsuperscript{1} with Asn\textsuperscript{1} in mouse and rat ET-2, which is also called VIC (4). The sequence of the carboxyl-terminal linear portion is shared by all of the members of the mammalian endothelin family; the amino acid substitutions between the isopeptides are clustered in the amino-terminai loop portions, especially within positions 2–7.

Endothelins have a wide variety of biological effects in many different target cell types. Their actions are mediated by specific cell surface receptors that belong to the superfamily of heptahelical G-protein-coupled receptors (5–7). Two subtypes of endothelin receptor, called ETA and ETB receptors, have been cloned, and both have been shown in many cell types to activate phospholipase C with resultant intracellular Ca\textsuperscript{2+} transients (8). However, the ETA and ETB receptors have distinct cell type/tissue distributions and thus have different physiological roles (9, 10). In many blood vessels, for example, the ETA receptors reside generally in smooth muscle cells and mediate vasoconstrictor responses, whereas the endothelial cells express the ETB receptor, which mediates vasodilator effects via the endothelin-induced release of nitric oxide. They can be pharmacologically distinguished by different rank orders of affinity toward endothelin isopeptides; the ETB receptor is ET-1-selective, showing an affinity rank order of ET-1 ≥ ET-2 >> ET-3, whereas the ETB receptor exhibits similar affinities to all three isopeptides. In other words, ET-1 can be considered as a nonselective agonist that exhibits similar subnanomolar affinities for both receptor subtypes. ET-3 is a moderately ETB-selective agonist with the affinity to the ETB receptor being 2 orders of magnitude higher than that to the ETA receptor. Recently, a number of synthetic ligands that are highly selective between endothelin receptor subtypes have been developed. For example, the cyclic pentapeptides BQ123, cyclo-(D-Trp-D-Asp-Pro-D-Val-Leu), and BQ153, cyclo-(D-Trp-D-Ala(SO\textsubscript{4})-Pro-D-Val-Leu), act as highly ETA-selective antagonists (11–13). Among highly ETB-selective agonists are BQ3020 (N-acetyl-[Ala\textsuperscript{1,16}]ET-1(16–21)) (14) and IRL1620 (N-succinyl-[Glu\textsubscript{6},Ala\textsuperscript{1,16}]ET-1(18–21)) (15).

Human ETA and ETB receptors exhibit a high polypeptide sequence identity to each other (\approx55\% overall; \approx74\% within the putative transmembrane helices) (16, 17). Since they maintain a clear distinction in ligand binding selectivity de-
spite this structural similarity, we set out to localize the subtype-specific determinants within endothelin receptors. We constructed a series of recombinant chimeras between human ET_A and ET_B receptors, expressed them in heterologous cell lines, and characterized those receptors in terms of ligand selectivity by competitive radioligand binding assays and agonist-induced intracellular Ca^{2+} transient assays. We found that the selectivity towards the ET_A-selective antagonist and the ET_B-selective agonist is specified by different subdomains of these receptors.

**MATERIALS AND METHODS**

Reagents—ET-1 and ET-3 were purchased from Peptide Institute (Osaka, Japan). BQ23 was a generous gift from Banyu Pharmaceutical Co., Ltd. (Tsukuba, Japan). 125I-ET-1 and 125I-BQ23 were purchased from Amersham Corp. 125I-IRL1620 was a kind gift from Dr. K. Maruyama of the Institute of Medical Science, University of Tokyo.

Chimeric Receptor Constructs—cDNA constructs encoding for chimeric human ET_A/ET_B receptors were assembled by creating common restriction sites within the wild-type receptor cDNAs (16, 17) by oligonucleotide-directed mutagenesis (18) and splicing the desired restriction fragments from the mutated cDNAs. Regions with well conserved amino acid sequences within the putative intracellular loops (ICL I-III) and extracellular loops (ECL I-III) were chosen for creating restriction sites. Care was taken to avoid any amino acid insertions/deletions and to minimize amino acid substitutions at the junction sites as much as possible. The restriction sites introduced and their positions in the deduced amino acid sequences of the ET_A/ET_B receptors, respectively, were as follows (see Fig. 1): SnaBI, at Ile^{100}/Ile^{101}; Apal, at Pro^{115}/Pro^{116}; BstBI, at Asp^{120}/Gly^{121}; BssHII, at Cys^{127}/Cys^{128}; Ncol, at Pro^{129}/Thr^{130}; BgII, at Glu^{132}/Glu^{133}; ClaI, at Arg^{140}/Arg^{141}. The conserved EcoRI sites at Asn^{170}/Asn^{171} within the seventh transmembrane helices (TM VII) of the wild-type receptors were also utilized. Where an amino acid substitution had to be introduced to create a restriction site, we confirmed that the ligand binding characteristics of the receptors carrying the point mutation alone were indistinguishable from those of the corresponding wild-type receptors (data not shown). The entire coding sequences of the wild-type and chimeric/mutant cDNAs were then subcloned into the SRa promoter-based mammalian expression vector pME18Sf—vector was a kind gift from Dr. K. Maruyama of the Institute of Medical Science, University of Tokyo.

**RESULTS**

Characterization of Wild-type Endothelin Receptors—COS-7 cells transfected with wild-type human ET_A and ET_B receptors cDNA constructs expressed specific binding sites for 125I-ET-1 (Fig. 2). The cells transfected with empty vector DNA had no detectable levels of specific ET-1 binding (data not shown). At the 125I-ET-1 concentration of 2 × 10^{-11} M, approximately 95.1 × 10^{10} molecules/cell of the radioligand were bound in the absence of competitors. The radioligand was displaced in a competitive manner by ET-1, ET-3, or BQ123. ET-1 had a similar binding affinity to both subtypes of receptor: apparent K_i values for the ET_A and ET_B receptors were 3.5 × 10^{-9} M and 9.5 × 10^{-10} M, respectively (Table I). ET-3 was virtually equipotent with ET-1 in displacing 125I-ET-1 from the ET_B receptor (K_i = 2.0 × 10^{-9} M), while being nearly 300 times less potent than ET-1 for the ET_A receptor (K_i = 1.0 × 10^{-8} M). BQ23 was highly selective for ET_A with K_i values for the ET_A and ET_B receptors of 2.5 × 10^{-10} M and 3.1 × 10^{-9} M, respectively. Ltk^- cells transiently transfected with the same constructs also showed virtually identical ligand binding characteristics. We detected no specific binding of...
FIG. 2. Displacement of $^{125}$I-ET-1 binding to COS-7 cells expressing the wild-type ETA receptor (panel A), the wild-type ETB receptor (panel B), and chimeric receptor A(N-III)B(IV-VI)A(VII-C) (panel C) by unlabeled ET-1 (O), ET-3 (O), and BQ123 (L). Levels of $^{125}$I-ET-1 binding are expressed as percentages of the specific binding in the absence of competitor.

Table I

Affinity of ET-1, ET-3, and BQ123 to wild-type human endothelin receptors determined by competitive binding assay with $^{125}$I-ET-1 as radioligand

| Receptor construct* | Affinity $K_i$ (nM) |
|---------------------|---------------------|
|                     | ET-1                | ET-3          | BQ123     | $R_{ET,3} = K_i(ET-3)/K_i(ET-1)$ | $R_{BQ123} = K_i(BQ123)/K_i(ET-1)$ |
| Wild-type B         | 0.95                | 2.0           | 31,000    | 2.1                              | 33,000                              |
| A(N)(II-C)          | 0.81                | 4.1           | 15,000    | 5.1                              | 18,000                              |
| A(N-DB)(II-C)       | 0.90                | 1.5           | 24,000    | 1.7                              | 27,000                              |
| A(N-III)(II-C)      | 0.83                | 4.8           | 8,900     | 5.8                              | 11,000                              |
| A(N-III)(IV-C)      | 1.7                 | 1.9           | 200       | 1.2                              | 120                                 |
| A(N-IV)(V-C)        | 1.0                 | 38            | 210       | 38                               | 210                                 |
| A(N-VI)(V-C)        | 1.0                 | 190           | 190       | 190                              | 130                                 |
| A(N-VI)(VII-C)      | 2.4                 | 1,100         | 190       | 450                              | 79                                  |
| A(N-VII)(B(C)       | 1.5                 | 930           | 9.6       | 610                              | 6.3                                 |
| Wild-type A         | 3.5                 | 1,000         | 25        | 290                              | 7.1                                 |
| B(N)(A-C)           | 1.3                 | 380           | 25        | 280                              | 19                                  |
| B(N-DA)(II-C)       | 1.4                 | 160           | 120       | 120                              | 85                                  |
| B(N-III)(A-C)       | 0.84                | 120           | 5,200     | 140                              | 6,100                               |
| B(N-III)(IV-C)      | 2.4                 | 1,000         | 50,000    | 420                              | 20,000                               |
| B(N-V)(V-C)         | 3.3                 | 1,300         | 51,000    | 390                              | 16,000                              |
| B(N-VI)(A-C)        | 0.93                | 51            | 35,000    | 55                               | 37,000                              |
| B(N-VI)(VII-C)      | 1.0                 | 6.2           | 41,000    | 6.0                              | 40,000                              |
| B(N-VII)(A(C)       | 1.2                 | 4.1           | 19,000    | 3.4                              | 16,000                              |
| A(N-III)(B(IV-VI)A(VII-C) | 1.0           | 1.1           | 20        | 1.1                              | 20                                  |
| B(N-IV)(A)(V)(VI-C) | 0.88                | 38            | 16,000    | 43                               | 19,000                              |
| B(N-DA-III)(B(IV-VI)A(VII-C)| 3.0           | ND            | 1,400     | ND                               | 480                                 |
| B(N-DA-III)(B(IV-VI)A(VII)(B(C)| 1.1           | ND            | 300       | ND                               | 270                                 |
| B(N)(A)(IV-V)(VI)(A)(B)(C)| 3.4           | ND            | 41        | ND                               | 12                                  |

* N, I-VII, and C designate the amino-terminal extracellular tail, transmembrane helices I-VII, and carboxyl-terminal cytoplasmic tail, respectively, plus the adjacent loop regions when applicable (see Fig. 1). A( . . . ) and B( . . . ) denote that the chimeric receptor has ETA and ETB sequences within the designated portions, respectively.

$R_{ET,3} = K_i(ET-3)/K_i(ET-1)$; $R_{BQ123} = K_i(BQ123)/K_i(ET-1)$.

ND, not determined.

FIG. 3. Dose-response relationships of $[^{45}]$Ca$^{2+}$ transients evoked by ET-1 (O) and ET-3 (O) in Ltk- cells expressing the wild-type ETA receptor (panel A), the wild-type ETB receptor (panel B), and chimeric receptor A(N-III)B(IV-VI)A(VII-C) (panel C). Initial peak $[^{45}]$Ca$^{2+}$ increments are plotted as percentages of the maximum $[^{45}]$Ca$^{2+}$ increments produced by ET-1 (10$^{-7}$ M) in cells expressing the wild-type ETA receptor.

$^{125}$I-ET-1 in Ltk- cells transfected with the empty vector DNA (data not shown).

ET-1 and ET-3 produced $[^{45}]$Ca$^{2+}$ transient responses in Ltk- cells transfected with the endothelin receptor constructs but not in the cells transfected with the empty vector plasmid. Fig. 3 shows the dose-response relationships for the initial peak increments of $[^{45}]$Ca$^{2+}$, evoked by ET-1 and ET-3 in the transfected Ltk- cells. ET-1 exhibited approximately equal potency toward both receptor subtypes; the agonist concentrations that elicited half-maximum response (EC$50$) for the ETA and ETB receptors were 1.3 x 10$^{-9}$ M and 4.9 x 10$^{-10}$ M, respectively. Although ET-3 was almost equipotent with ET-
1 toward the ET$_B$ receptor (EC$_{50} = 6.0 \times 10^{-10}$ M), it was 25 times less potent than ET-1 for ET$_A$ (EC$_{50} = 3.3 \times 10^{-8}$ M). Furthermore, ET-3 produced a considerably smaller maximum response for ET$_A$-expressing cells as compared with ET-1; the maximum responses to ET-3 (at 10$^{-7}$ M) were 28 and 100% of those to ET-1 in the cells expressing the ET$_A$ and ET$_B$ receptors, respectively. In the ET$_A$-expressing cells, BQ123 (10$^{-6}$ M) inhibited an ET-1 (10$^{-6}$ M)-induced [Ca$^{2+}$] response by only 9% in the cells expressing the ET$_A$ receptor. BQ123 at up to 10$^{-4}$ M had no detectable agonist activity for either receptor subtype in this assay (data not shown).

Characterization of Chimeric Receptors—We constructed two systematic series of chimeric endothelin receptors by progressively substituting the structure of the ET$_A$ receptor with that of the ET$_B$ receptor (see “Materials and Methods”). The progressive substitutions from the amino-terminal extracellular tail resulted in the series of A/B chimeras, whereas the amino-terminal substitutions gave rise to B/A chimeras. Thus, for example, chimera A(N-II)B(III-C) hereafter designates a chimeric receptor consisting of the ET$_A$ sequence from the amino-terminal putative extracellular tail through TM II, followed by the ET$_B$ sequence from the TM III through the carboxy-terminal cytoplasmic tail. Each chimeric molecule was expressed in COS-7 cells, and the cells were subjected to a competitive radioligand binding assay. All chimeric receptor constructs we tested conferred similar densities of $^{125}$I-ET-1 binding sites in these cells; between 0.5 and 1 $\times$ 10$^7$ molecules/cell of $^{125}$I-ET-1 (at 2 $\times$ 10$^{-11}$ M) were bound in the absence of competitors. Table I summarizes the apparent $K_i$ values of the competitors determined for the wild-type and chimeric receptors expressed in COS-7 cells. The $K_i$ values for ET-1 were similar in the wild-type and all chimeric receptors, all being within the range from 8.1 $\times$ 10$^{-10}$ M to 3.5 $\times$ 10$^{-9}$ M. Provided with this uniformity of the affinity to ET-1 in all chimeric receptors, we divided the $K_i$ values for ET-3 and BQ123 by those for ET-1 in each receptor construct and used these affinity ratios (designated hereafter as $R_{ET-3}$ and $R_{BQ123}$, respectively) as an indicator of the ligand selectivity exhibited by each recombinant receptor (Table I).

Determinant for Selectivity to BQ123—Replacement of the amino-terminal extracellular tail of ET$_A$ with the corresponding region of ET$_B$ caused little change in the affinity to BQ123 (chimera B(N)A(I-C)). However, further progressive replacement of the TM I, II, and III of ET$_A$ (including the intervening loops) with the corresponding regions from ET$_B$ resulted in a progressive increase in $K_i$ values for BQ123 and thus in $R_{BQ123}$ values (chimeras B(N-I)A(II-C), B(N-II)A(III-C), and B(N-III)A(IV-C)). Replacement of the carboxy-terminal half of TM VII together with the carboxy-terminal cytoplasmic tail of ET$_A$ with the corresponding region of ET$_B$ caused no significant change in BQ123 selectivity (chimera A(N-VII)B(C)). Further substitution of the amino-terminal half of TM VII plus the carboxy-terminal half of ECL III resulted in an 11-fold increase in the $R_{BQ123}$ value (chimera A(N-VI)B(VII-C)). The selectivity to BQ123 did not change very much when further substitution of the ET$_A$ sequence from the carboxy-terminal side was carried out through TM IV and ICL II (chimeras A(N-IV)B(VI-C), A(N-IV)B(V-C), and A(N-III)B(IV-C)). However, when the TM III together with the carboxy-terminal half of ECL I was further substituted (chimera A(N-II)B(III-C)), an additional ~100-fold increase of $R_{BQ123}$ was observed.

These results suggest that the ET$_A$ sequences spanning from TM I through TM III as well as the amino-terminal half of TM VII are likely to be important for its high affinity binding to BQ123. To test whether these regions were also sufficient to define the binding determinant for the ET$_A$-selective antagonist, we constructed the chimeras B(N-I)B(II-VI)A(III-C). This chimeric receptor exhibited the ability to bind BQ123 with high affinity, with an $R_{BQ123}$ value similar to that for the wild-type ET$_A$ receptor (Table I and Fig. 6). Furthermore, BQ123 (10$^{-6}$ M) inhibited the ET-1-induced [Ca$^{2+}$] transient response by 96% in Ltk$^{-}$ cells transfected with this chimeric construct (Fig. 4). BQ123 showed no detectable agonist activity at up to 10$^{-4}$ M. Although the chimeras B(N-I)B(II-VI)A(III-C) maintained high affinity to BQ123, two additional chimeras (B(N-I)A(II-III)B(IV-C) and B(N-I)A(II-III)B(IV-VI)A(VII)B(C)) exhibited intermediate binding affinities to BQ123, indicating that the structures of TM I and TM VII from the ET$_A$ receptor indeed contributed to the high affinity binding of BQ123.

Determinant for Selectivity to ET-3—Progressive replacement of the sequence of the ET$_A$ receptor with that of ET$_B$ from the amino-terminal extracellular tail through TM III caused little changes in $R_{ET-3}$ values (chimeras A(N-I)B(I-C), A(N-I)B(II-C), A(N-I)B(III-C), and A(N-I)B(IV-C)). However, when the ICL II and TM IV were further substituted with the corresponding parts of ET$_A$, the $R_{ET-3}$ value increased to 38 (chimera A(N-IV)B(V-C)). Further substitution with the ET$_A$ sequence made the resultant chimeras indistinguishable.
from the wild-type ETA receptor in terms of $R_{ET-3}$ values. Similarly, progressive replacement of the ETA sequence with ETB from the carboxyl-terminal cytoplasmic tail through the carboxyl-terminal half of ECL III (chimeras B(N-III)A(VI-C)) and B(N-III)A(VI-C) resulted in little change in $R_{ET-3}$. However, further substitution of the amino-terminal half of ECL III, TM VI, and most of ICL III with the ETB sequence caused a significant increase of the selectivity ratio to 55 (chimera B(N-III)B(IV-VI)A(VII-C)).

These results suggest that the structure of the ETB receptor spanning ICL II through the amino-terminal half of ECL III was necessary to render the receptor able to bind ET-3 with high affinity. To confirm further that the above mentioned region from the ETB receptor is sufficient to provide high affinity binding of ET-3, we constructed the chimera A(N-III)B(IV-VI)A(VII-C). Indeed, this chimeric receptor had virtually equal affinities for ET-1 and ET-3 both in the radioligand binding assay and in the [Ca$^{2+}$]$_i$ transient assay (Table I and Figs. 2 and 3). In contrast, the chimeric receptor B(N-IV)A(VI-C) displayed an intermediate affinity to ET-3; its $R_{ET-3}$ value was 7 times lower than the wild-type ETA yet 20 times higher than the ETB. This indicates that although the sequence of the ETB receptor spanning ECL II and TM V significantly contributes to the binding determinant for ET-3, this structure alone is not sufficient to form the complete determinant.

There are two charge-modifying amino acid substitutions between the ETA and ETB receptors in their TM IV and TM VI; namely, Asp$^{376}$ and Leu$^{247}$ in ETB versus Val$^{376}$ and Lys$^{247}$ in ETA. To examine if these substitutions of charged residues have any effect on the binding affinity of ET-3, we introduced the following point missense mutations at these positions: ETA(D241V), ETA(L347K), ETA(D241V/L347K), ETA(V225D), ETA(K330I), ETA(V225D/K330I). However, we found that the ET-3 selectivities of these mutant receptors were all indistinguishable from the respective wild-type receptors (data not shown).

A Chimeric Receptor with High Affinity to Both ETA and ETB-selective Ligands—Since TM I, II, III and VII from the ETA receptor (including the intervening loop regions) were sufficient to form the high affinity binding determinant for BQ123 in the chimeric receptors, we expected that the "ETB-like" chimera A(N-III)B(IV-VI)A(VII-C) could still maintain high affinity to BQ123. This was actually the case; the chimeric receptor had an affinity to BQ123 which was very similar to native ETA receptor (Table I and Fig. 2). Therefore, it seemed that this particular chimeric receptor could accept both ETA- and ETB-selective ligands with high affinity. We performed competitive binding studies on this chimeric construct by using the highly ETA-selective agonists $^{35}$S-BQ3020 and $^{35}$S-IRL1620 as radioligands and the highly ETA-selective antagonist BQ123 as competitor. We found that the chimeric receptor specifically bound these ETA-selective radioligands in a manner similar to the native ETA receptor (Fig. 6). Moreover, the specific binding of the ETB-selective radioligands was completely abolished by 10$^{-8}$ M BQ123 in the chimeric receptor (Fig. 6). In contrast, in the case of the wild-type ETA receptor, the radioligand binding was affinity by 10$^{-4}$ M BQ123 only slightly. The apparent $K_i$ values for BQ123 in displacing the specific binding of $^{35}$S-BQ3020 and $^{35}$S-IRL1620 in the chimeric receptor were 1.9 $\times$ 10$^{-9}$ M and 1.0 $\times$ 10$^{-8}$ M, respectively (Fig. 7). These values were similar to the $K_t$ values for BQ123 observed in the wild-type ETA receptor by using $^{35}$S-ET-1 as radioligand (Table I and Fig. 2).

We further examined whether BQ123 still acted as an antagonist for the chimeric receptor A(N-III)B(IV-VI)A(VII-C) as radioligand (Table I and Fig. 2).
peptide ligand families such as tachykinins (24).

The amino acid sequences of the ETA and ETB receptors are most dissimilar to each other in their putative amino-terminal extracellular tails, which have predicted N-glycosylation sites. In fact, there is no detectable sequence similarity between these portions of the two receptor subtypes at all. It is therefore somewhat surprising that mutual swapping of the extracellular tails between the ETA and ETB receptors had no appreciable effect on their ligand binding characteristics (chimeras B(N)A(I-C) and A(N)B(I-C)). The function of these parts of the receptor molecules is not clear at present; one possibility is that they may assist the receptor polypeptides to fold and be expressed on the cell surface in the proper transmembrane orientations.

BQ123 is an established competitive antagonist selective for the ETA receptor (11). The three-dimensional structures of BQ123 and ET-1 in solution have been demonstrated with proton NMR by several independent laboratories (25–28). However, so far it has been unclear as to which part of the ET-1 structure may be mimicked by the antagonist. We demonstrated in this study that the chimeric receptor A(N-III)(B(IV–VI))A(VII–C) could achieve a high affinity binding of both BQ123 and the two ETB-selective agonists, BQ3020 and IRL1620. Furthermore, we found that BQ123 could compete with iodinated BQ3020 or IRL1620 for the specific binding to this chimeric receptor. The latter ligands are linear, amino-terminally truncated derivatives of ET-1. These findings strongly argue for the idea that BQ123 mimics a structure within the carboxyl-terminal, linear portion of ET-1. In this regard, the amino acid sequence of BQ123 appears to have certain similarity to the carboxyl-terminal sequence of ET-1. The motif found in BQ123, -d-Val-Leu-d-Trp-d-Asp, may mimic the carboxyl terminus of endothelins, -Ile-Ile-Trp-COOH. We also demonstrated that the determinant for the high affinity binding of BQ23 resides within the TM I–III and VII (including adjacent loops) of the ETA receptor. This is in agreement with the findings recently reported by Adachi et al. (29) that the ECL I from the ETA receptor is an important determinant for BQ23 binding. Taken together with the above argument, it seems plausible to consider that these portions of the receptor molecules contain at least a part of their binding domain for the carboxyl termini of endothelins, which includes the Trp residue essential for the binding of the peptide to either receptor subtype (30).

We have demonstrated that the TM IV–VI and the adjacent loop regions of the ETB receptor constitute the high affinity binding determinant for the ETB-selective agonists, including the amino-terminally truncated linear ET-1 derivatives. This is in sharp contrast to the case of the heptahelical receptors for tachykinins, in which the region spanning from TM II to ECL II (together with a minor contribution of the amino-terminal tail) has been recently reported to specify isopeptide selectivity (24). It is interesting to note that, like endothelin family, tachykinins have a common carboxyl-terminal motif and divergent amino-terminal portions. These observations indicate a significant difference in the mode of receptor/ligand interactions between the endothelin and tachykinin systems, despite these apparent similarities in the general configurations of isopeptides and receptors.

The findings presented in this study provide further insight into the possible mechanism for the ligand selectivity of endothelin receptors. It is plausible to consider that the structures of endothelin isopeptides and their derivatives are comprised of two distinct subdomains: the amino-terminal disulfide loop portion that is variable among isopeptides, and the carboxyl-terminal linear hydrophobic region that is highly conserved. The currently available information on the structure-activity relations of endothelin derivatives suggests the following general rules (30, 31): (i) both the amino-terminal loop structure from ET-1 (or other nonselective ligands such as sarafotoxin S6b) and the common carboxyl-terminal linear structure are required for high affinity binding to the ETA receptor; (ii) in contrast, the ETB receptor only requires the carboxyl-terminal half of the ligand. In this context, the present results suggest that the TM IV–VI of endothelins may interact with the amino-terminal loop portion of the ligands. Incorporating these considerations, we present a hypothetical model for the binding determinants of endothelin receptors and their ligands (Fig. 8). Thus, it is conceivable that the amino-terminal loop domain of ET-1 functions in a manner similar to a classical “address” domain suggested for a number of peptide ligand family (32). The TM IV–VI of the ETA receptor interacts selectively with this address domain of ET-1 to promote a full activation of receptor, which is brought about by the carboxyl-terminal “message” domain of ET-1. However, the corresponding address domains of the ETB-selective ligands are either invalid (ET-3) or missing (BQ3020, IRL1620), resulting in the inability to interact with the ETB receptor. In contrast, the ETB receptor, with its subtly different structures within the TM IV–VI region, may be highly promiscuous for the ligands’ address domains. Alternatively, it is tempting to speculate that the TM IV–VI domain of the ETB receptor may function as a self-contained address recognition domain that does not require a valid address information presented by the ligand. Further, it is also conceivable that the mode of interaction between the cyclic pentapeptide antagonist BQ123 and the ETA receptor...
is significantly different from agonist/receptor interactions so that it does not require the interaction at the addressing domain.

Acknowledgments—We thank Drs. Mitsuo Yano and Masaki Ihara of Banyu Pharmaceutical Co. Ltd. for samples of BQ123. We also thank Dr. Kazuo Maruyama of the Institute of Medical Science, University of Tokyo, and Dr. Beverly Brown of Du Pont-New England Nuclear for the expression vector pME18Sf- and iodinated it does not require the interaction at the addressing domain.

REFERENCES

1. Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K., and Masaki, T. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2863-2867
2. Masaki, T., Yanagisawa, M., and Goto, K. (1992) Med. Res. Rev. 12, 391-421
3. Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsu, Y., Yatani, Y., Goto, K., and Masaki, T. (1988) Nature 332, 411-415
4. Saida, K., and Mitsui, Y. (1991) J. Cardiovasc. Pharmacol. 17, (Suppl. 7) 55-58
5. Jackson, T. (1991) Pharmacol. Ther. 50, 425-442
6. Dohman, H. G., Thorner, J., Caron, M. G., and Lefkowitz, R. J. (1991) Annu. Rev. Biochem. 60, 653-686
7. Strousberg, A. D. (1991) Eur. J. Biochem. 196, 1-10
8. Sakurai, T., Yanagisawa, M., and Masaki, T. (1992) Trends Pharmacol. Sci. 13, 105-108
9. Horii, S., Komatsu, Y., Shigemoto, R., Mizuno, N., and Nakanishi, S. (1992) Endocrinology 130, 1855-1896
10. Nakamichi, K., Ihara, M., Kobayashi, M., Sae, T., Ishikawa, K., and Yano, M. (1992) Biochem. Biophys. Res. Commun. 182, 144-150
11. Ihara, M., Suguchi, R., Sae, T., Fukurada, T., Tsuchida, S., Kimura, S., Fukum, T., Ishikawa, K., Nishikibe, M., and Yano, M. (1992) Life Sci. 50, 247-255
12. Ishikawa, K., Fukum, T., Nagase, T., Fujita, K., Hayama, T., Niyama, K., Mase, T., Ihara, M., and Yano, M. (1992) J. Med. Chem. 35, 2139-2142
13. Fukurada, T., Nishikibe, M., Ohta, Y., Ihara, M., Yano, M., Ishikawa, K., Fukum, T., and Iizomoto, F. (1992) Life Sci. 50, PL107-PL112
14. Ihara, M., Sae, T., Fukurada, T., Kimura, S., Ozaki, S., Patel, A. C., and Yano, M. (1992) Life Sci. 51, PL1-PL52
15. Watanabe, T., Ura, Y., Takai, M., Uemura, I., and Okada, T. (1992) Biochem. Biophys. Res. Commun. 185, 867-873
16. Hosoda, K., Nakao, K., Arai, H., Suga, S., Ogawa, Y., Mukoyama, M., Shirakami, G., Saito, Y., Nakanishi, S., and Imura, H. (1991) FEBS Lett. 287, 23-26
17. Sakamoto, A., Yanagisawa, M., Seki, T., Fukushima, T., and Masaki, T. (1991) Biochem. Biophys. Res. Commun. 178, 665-663
18. Kunkel, T. A. (1980) Proc. Natl. Acad. Sci. U. S. A. 8192, 488-492
19. Takabe, Y., Seki, M., Fujisawa, J., Hori, T., Yokota, K., Arai, K., Yoshida, M., and Arai, N. (1986) Mol. Cell. Biol. 6, 466-472
20. Sakurai, T., Yanagisawa, M., Takai, M., Nakanishi, K., and Masaki, T. (1990) Nature 348, 132-135
21. Grynkiewicz, G., Pide, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3430-3435
22. DeLean, A., Munson, P. J., and Rodbard, D. (1979) Am. J. Physiol. 235, 97-102
23. Vegeto, E., Allan, G. F., Schnader, W. T., Tasi, M. J., McDonnell, D. P., and O'Malley, B. W. (1992) Cell 70, 703-713
24. Yokota, Y., Akazawa, C., Ohkubo, H., and Nakanishi, S. (1992) EMBO J. 11, 3385-3391
25. Atkinson, R. A., and Felton, J. T. (1992) FEBS Lett. 296, 1-6
26. Krytske, S. R., Jr., Bassolino, D. A., Brucoleri, R. E., Hunt, J. T., Jr., and Rodrigo, P. L. (1992) FEBS Lett. 299, 255-261
27. Reilly, M. D., Thanabel, V., Omacinsky, D. O., Donahue, J. B., Jr., Doherty, A. M., and DePue, P. L. (1992) FEBS Lett. 300, 136-140
28. Andersen, N. H., Chen, C. P., Molschner, T. M., Krystek, S. R., Jr., and Bassolino, D. A. (1992) Biochemistry 31, 1284-1290
29. Adachi, M., Yang, Y.-Y., Tazaki, A., Furui, Y., and Miyamoto, M. (1992) FEBS Lett. 311, 179-183
30. Doherty, A. M. (1992) J. Med. Chem. 35, 1495-1508
31. Ura, Y., Fujita, Y., Oda, K., Watanabe, T., Uemura, I., Takai, M., Okada, Y., Sakata, K., and Kusak, H. (1992) FEBS Lett. 311, 12-16
32. Tsuda, A. E., and Fortgese, P. S. (1992) Ann. Rev. Pharmacol. Toxicol. 32, 239-269