Vol. 177, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1991 Pages 34-39

CLONING AND SEQUENCE ANALYSIS OF A cDNA ENCODING HUMAN NON-SELECTIVE TYPE OF ENDOTHELIN RECEPTOR

Makoto Nakamuta[1], Ryoichi Takayanagi[1*], Yoshiyuki Sakai[1], Shigeru Sakamoto[1], Hiromi Hagiwara[2], Takeshi Mizuno [2], Yasuyoshi Saito[2], Shigehisa Hirose[2], Mikio Yamamoto[3] and Hajime Nawata[1]

[1] The Third Department of Internal Medicine, Faculty of Medicine,Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812, Japan

[2] Department of Biological Sciences, Tokyo Institute of Technology, Ookayama, Meguro-ku, Tokyo 152, Japan

[3] The Second Department of Biochemistry, National Defense Medical College, Tokorozawa 359, Japan

Received April 15, 1991

Summary: A cDNA encoding non-selective type (ET_B) of endothelin receptor was isolated from a human liver cDNA library. The cDNA had an open reading frame encoding a protein of 442 amino acid residues with a relative Mr of 49,643. The deduced amino acid sequence of human ET_B receptor was 88% and 64% identical to those of rat lung ET_B receptor and bovine lung ET-1-specific (ET_A) receptor, respectively, and contained a relatively long and proline-rich extracellular N-terminal region in addition to a significant similarity with the G protein-coupled receptor superfamily with seven transmembrane segments.

Endothelins (ETs) belong to a potent vasoactive peptide family consisting of three isopeptides, ET-1, ET-2 and ET-3 (1). A diverse set of pharmacological activities with different potencies exerted by ET family peptides (1,2,3) suggested the existence of ET receptor subtypes. We have reported that two distinct subclasses of ET receptors, namely, ET-1-specific and ET family-common receptors, are distributed in various proportions in human and porcine tissues (4). Most recently, the structure of the ET-1-specific receptor of bovine lung (5) and that of the non-selective receptor of rat lung (6) have been determined by cloning their cDNAs, and it was recommended that the ET-1-specific type be called ET_A and the non-selective one ET_B (7).

* To whom correspondence should be addressed.

0006-291X/91 $1.50

In the present study, to clarify the structure of human ET receptor, we have screened and isolated a cDNA encoding human ET_B receptor from a cDNA library constructed with human liver, since hepatocytes have a considerable amount of ET receptors linked to biological actions such as glycogenolysis (8).

Materials and Methods

Materials: Adult human intact liver was obtained at surgery from a patient with hepatolithiasis. A mixture of partially degenerated oligonucleotide probes corresponding to the tryptic fragments NH_2-AlaAsnAspHisGlyTyrAspAsnPhe-COOH and NH_2-SerGly-MetGlnIleAlaLeuAsnAspHisLeuLys-COOH were synthesized on the basis of amino acid sequences of tryptic fragments of previously purified bovine lung endothelin receptors (9) and used for screening.
Construction and screening of a human liver cDNA library: Total RNA was prepared from human liver by a phenol/SDS method (10), and poly(A)$^+$RNA was purified by chromatography on oligo(dT)-cellulose. A cDNA library was constructed using a mixture of random oligodeoxyhexamer (Boehringer, Mannheim, Germany) for the primer of reverse transcription and commercially available kits for cDNA synthesis (Pharmacia LKB Biotechnology, Uppsala, Sweden), λZAPII cloning and λ-DNA in vitro packaging (Stratagene, CA, USA). This cDNA library was screened using ^{32}P-labeled 27-mer and 36-mer oligonucleotide probes by a plaque hybridization technique (10).
Determination of nucleotide sequence: An ordered set of subclones deleted from both termini of a cDNA insert was constructed by Bal 31 nuclease-digestion (Takara Shuzo CO. LTD., Kyoto, Japan) following subcloning into SmaI site of pUC118. Nucleotide sequences of both plus and minus strands were determined by dideoxy chain termination method (11).
Northern blot analysis: Poly(A)$^+$RNA (10μg) purified from human liver were separated by formaldehyde/1.5% agarose gel electrophoresis, and transferred to a nitrocellulose membrane. Hybridization was carried out in the presence of 50% formamide at 42° C for 12 h, using the entire insert of pHETR-4 as a probe. The membrane was washed with 0.1xSSC containing 0.1% SDS at 50° C for 20 min and autoradiographed overnight at -70° C.

Results and Discussion

We have determined the amino acid sequences of several tryptic fragments obtained from purified bovine lung ET receptor (9). Two oligonucleotide probes which are complementary to the sequences coding the two fragments chosen (see Materials and Methods) were synthesized. By screening the human liver cDNA library constructed in λZAPII, ten clones positive with both the probes were isolated from $4x10^6$ clones. The sequence analysis of these clones revealed that one clone, pHETR-4, with a 1,470 bp insert contained the longest 1326 bp open reading frame encoding 442 amino acid residues with a relative molecular mass of 49,643 (Fig.1), which begins with a basic and hydrophobic 26 amino acid

```
                                                GAAACTGCGGACGGGCCACCGGACGCCTTCTGGAGCAGGTAGCAGC    -1

ATGCAGCCGCCTCCAAGTCTGTGCGGACGCGCCCTGGTTGCGCTGGTTCTTGCCTGCGGCCTGTCGCGGATCTGG    75
MetGlnProProProSerLeuCysGlyArgAlaLeuValAlaLeuValLeuAlaCysGlyLeuSerArgIleTrp    25

GGAGAGGAGAGAGGCTTCCCGCCTGACAGGGCCACTCCGCTTTTGCAAACCGCAGAGATAATGACGCCACCCACT   150
GlyGluGluArgGlyPheProProAspArgAlaThrProLeuLeuGlnThrAlaGluIleMetThrProProThr    50

AAGACCTTATGGCCCAAGGGTTCCAACGCCAGTCTGGCGCGGTCGTTGGCACCTGCGGAGGTGCCTAAAGGAGAC   225
LysThrLeuTrpProLysGlySerAsnAlaSerLeuAlaArgSerLeuAlaProAlaGluValProLysGlyAsp    75

AGGACGGCAGGATCTCCGCCACGCACCATCTCCCCTCCCCCGTGCCAAGGACCCATCGAGATCAAGGAGACTTTC   300
ArgThrAlaGlySerProProArgThrIleSerProProProCysGlnGlyProIleGluIleLysGluThrPhe   100

AAATACATCAACACGGTTGTGTCCTGCCTTGTGTTCGTGCTGGGGATCATCGGGAACTCCACACTTCTGAGAATT   375
LysTyrIleAsnThrValValSerCysLeuValPheValLeuGlyIleIleGlyAsnSerThrLeuLeuArgIle   125

ATCTACAAGAACAAGTGCATGCGAAACGGTCCCAATATCTTGATCGCCAGCTTGGCTCTGGGAGACCTGCTGCAC   450
IleTyrLysAsnLysCysMetArgAsnGlyProAsnIleLeuIleAlaSerLeuAlaLeuGlyAspLeuLeuHis   150

ATCGTCATTGACATCCCTATCAATGTCTACAAGCTGCTGGCAGAGGACTGGCCATTTGGAGCTGAGATGTGTAAG   525
IleValIleAspIleProIleAsnValTyrLysLeuLeuAlaGluAspTrpProPheGlyAlaGluMetCysLys   175

CTGGTGCCTTTCATACAGAAAGCCTCCGTGGGAATCACTGTGCTGAGTCTATGTGCTCTGAGTATTGACAGATAT   600
LeuValProPheIleGlnLysAlaSerValGlyIleThrValLeuSerLeuCysAlaLeuSerIleAspArgTyr   200

CGAGCTGTTGCTTCTTGGAGTAGAATTAAAGGAATTGGGGTTCCAAAATGGACAGCAGTAGAAATTGTTTTGATT   675
ArgAlaValAlaSerTrpSerArgIleLysGlyIleGlyValProLysTrpThrAlaValGluIleValLeuIle   225

TGGGTGGTCTCTGTGGTTCTGGCTGTCCCTGAAGCCATAGGTTTTGATATAATTACGATGGACTACAAAGGAAGT   750
TrpValValSerValValLeuAlaValProGluAlaIleGlyPheAspIleIleThrMetAspTyrLysGlySer   250

TATCTGCGAATCTGCTTGCTTCATCCCGTTCAGAAGACAGCTTTCATGCAGTTTTACAAGACAGCAAAAGATTGG   825
TyrLeuArgIleCysLeuLeuHisProValGlnLysThrAlaPheMetGlnPheTyrLysThrAlaLysAspTrp   275

TGGCTGTTCAGTTTCTATTTCTGCTTGCCATTGGCCATCACTGCATTTTTTTTATACACTAATGACCTGTGAAATG   900
TrpLeuPheSerPheTyrPheCysLeuProLeuAlaIleThrAlaPhePheTyrThrLeuMetThrCysGluMet   300

TTGAGAAAGAAAAAGTGGCATGCAGATTGCTTTAAATGATCACCTAAAGCAGAGACGGGAAGTGGCCAAAACCGTC   975
LeuArgLysLysSerGlyMetGlnIleAlaLeuAsnAspHisLeuLysGlnArgArgGluValAlaLysThrVal   325

TTTTGCCTGGTCCTTGTCTTTGCCCTCTGCTGGCTTCCCCTTCACCTCAGCAGGATTCTGAAGCTCACTCTTTAT  1050
PheCysLeuValLeuValPheAlaLeuCysTrpLeuProLeuHisLeuSerArgIleLeuLysLeuThrLeuTyr   350

AATCAGAATGATCCCAATAGATGTGAACTTTTGAGCTTTCTGTTGGTATTGGACTATATTGGTATCAACATGGCT  1125
AsnGlnAsnAspProAsnArgCysGluLeuLeuSerPheLeuLeuValLeuAspTyrIleGlyIleAsnMetAla   375

TCACTGAATTCCTGCATTAACCCAATTGCTCTGTATTTGGTGAGCAAAAGATTCAAAAACTGCTTTAAGTCATGC  1200
SerLeuAsnSerCysIleAsnProIleAlaLeuTyrLeuValSerLysArgPheLysAsnCysPheLysSerCys   400

TTATGCTGCTGGTGCCAGTCATTTGAAGAAAAACAGTCCTTGGAGGAAAAGCAGTCGTGCTTAAAGTTCAAAGCT  1275
LeuCysCysTrpCysGlnSerPheGluGluLysGlnSerLeuGluGluLysGlnSerCysLeuLysPheLysAla   425

AATGATCACGGATATGACAACTTCCGTTCCAGTAATAAATACAGCTCATCTTGAAAGAAGAACTATTCACTGTAT  1350
AsnAspHisGlyTyrAspAsnPheArgSerSerAsnLysTyrSerSerSer *                          442

TTCATTTTCTTTATATTGGACCGAAGTCATTAAAACAAAATGAAACATTTGCCAAAACAAAACAAAAAACTATG   1424
```

Fig.1. Nucleotide and deduced amino acid sequence of human ET_B receptor cDNA cloned in pHETR-4. The predicted 442-residue amino acid sequence from the first ATG is shown below the nucleotide sequence. The predicted cleavage site of the secretory signal sequence is indicated by an arrow. One potential N-glycosylation site is indicated by a dot. Boxes show the amino acid sequences of tryptic fragments of the purified bovine lung ET receptor. Serine residues potentially able to be phosphorylated are underlined.

sequence, putatively defined as a signal peptide. The surrounding sequence of the ATG initiation triplet agrees well with the consensus sequence (12). The validity of pHETR-4 as an ET receptor-encoding clone was supported by the presence of the amino acid sequences of tryptic fragments of the purified bovine lung ET receptor (the boxed region in Fig.1). One of the other clone, pHETR-2, contained a 2,152 bp insert beginning at position 7 of

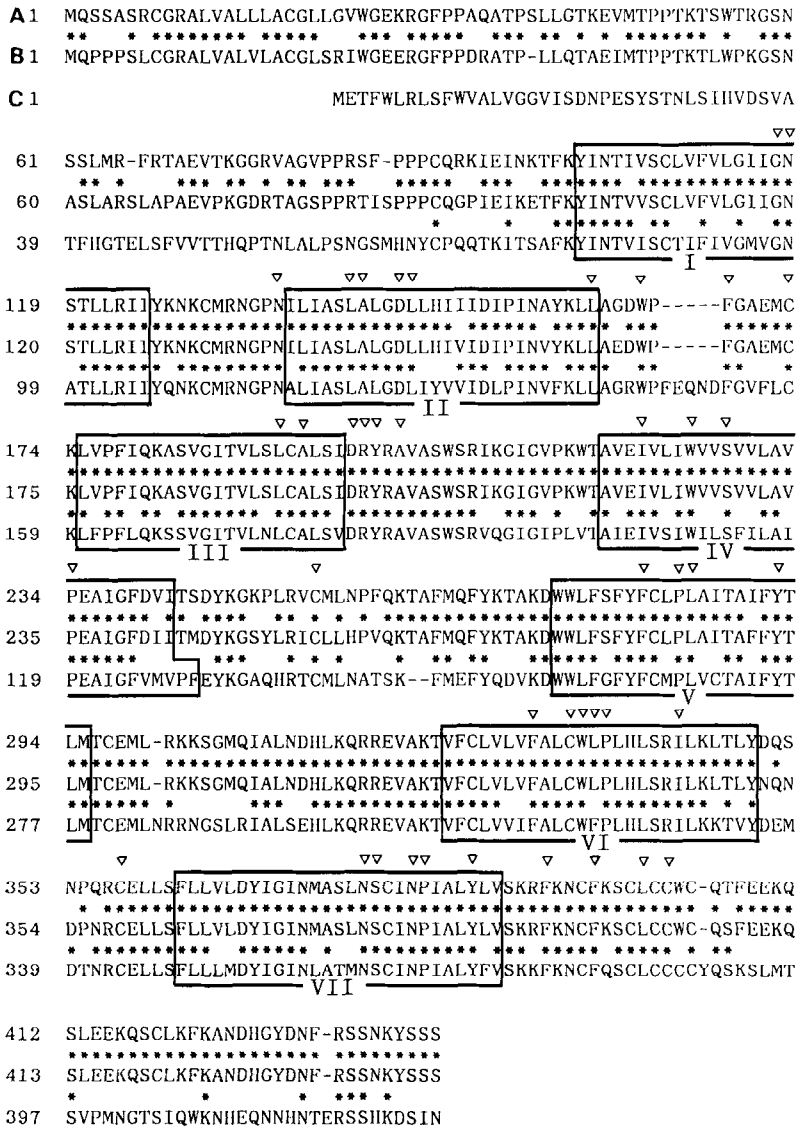

Fig.2. Homology in the deduced amino acid sequences of human ET_B, rat ET_B and bovine ET_A receptors. The sequences are shown by single-letter code. A, rat lung ET_B receptor (6); B, human liver ET_B receptor; C, bovine lung ET_A receptor (5). Amino acids of rat or bovine ET receptor identical to those of human receptor are indicated by stars. The putative transmembrane domains I-VII are boxed. Highly conserved amino acid residues in the G protein-coupled receptor superfamily are indicated by symbols, ▽.

the open reading frame of a pHETR-4 insert, and the cDNA sequences of the two clones were identical in their overlapped regions.

As shown in Fig.2, the deduced structure of human liver ET receptor exhibits a significant similarity with G protein-coupled receptors, and contains seven hydrophobic segments corresponding

to transmembrane domains (the boxed region in Fig.2) and highly conserved residues in the G protein-coupled receptor superfamily, as reported in the structures of bovine lung ET_A (5) and rat lung ET_B (6) receptors. One consensus site for N-glycosylation (Asn^{59}) and several serine residues for a potential phosphorylation site are also present in human liver ET receptor (Fig.1). The deduced amino acid sequence of human liver ET receptor was 88% identical to that of the rat ET_B receptor but only 64% to that of the bovine ET_A receptor. A relatively long extracellular N-terminal region of human liver ET receptor, which may be involved in ligand selectivity, has no homology with that of the ET_A receptor, and is appreciably rich in proline residues, being homologous to the N-terminal region of the ET_B receptor. These findings strongly suggested that the human liver ET receptor cloned in the present study belongs to an ET_B type receptor. Indeed, COS cells transfectecd with an expression vector of pCMV2 (13) containing a cDNA insert of pHETR-4 showed almost an equal affinity for every ET isopeptides (manuscript in preparation). The amino acid sequence of human ET_B receptor was one amino acid longer than that of rat ET_B receptor.

Northern blot analysis of poly(A)$^+$ RNA from human liver using the pHETR-4 insert as a hybridization probe showed the presence of an approximately 5.0 kb receptor mRNA, the molecular size of which is in good agreement with that of rat ET_B receptor (6) (Fig. 3).

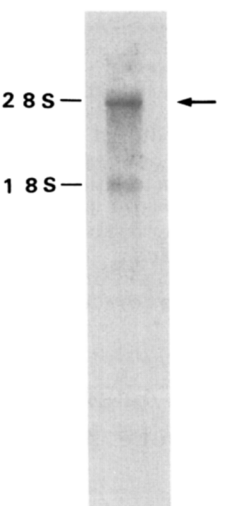

Fig.3. Northern blot analysis of poly(A)$^+$RNA (10μg) from human liver with pHETR-4 insert as a probe. The approximately 5.0 kb ET_B receptor mRNA is indicated by an arrow.

The present study provided the complete amino acid structure of human ET_B receptor, which may facilitate the identification of other possible ET receptor subtypes and also the development of a new type of vasodilators, because ET_B is linked to vasodilation (14).

Acknowledgment: This study was in part supported by a Grant-in-Aid for General Scientific Research from The Ministry of Education, Science and Culture, Japan.

References

1. Yanagisawa,M. and Masaki,T. (1989) Trends Pharmacol. Sci. (review), 10. 374-378.
2. Inoue,A., Yanagisawa,M. ,Kimura,S., Kasuya,Y., Miyauchi,T., Goto,K. and Masaki,T. (1989) Proc. Natl. Acad. Sci. USA, 86, 2863-2867.
3. Warner,T.D., DeNucci,G. and Vane,J.R. (1989) Eur. J. Pharmacol., 159, 325-326.
4. Takayanagi,R., Ohnaka,K., Takasaki,C., Ohashi,M. and Nawata,H. (1991) Regul. Pep., 32, 23-37.
5. Arai,H., Hori,S., Aramori,I., Ohkubo,H. and Nakanishi,S. (1990) Nature, 348, 730-732.
6. Sakurai,T., Yanagisawa,M., Takuwa,Y., Miyazaki,H., Kimura,S., Goto, K. and Masaki,T. (1990) Nature, 348, 732-735.
7. Vane,J.R. (1990) Nature, 348, 673.
8. Serradeli-LeGal,C., Jouneaux,C., Sanchez-Bueno,A., Raufaste,D., Roche,B., Prēaux,A.M., Maffrand,J.P., Cobbold,P.H., Hanoue,J. and Lotersztajin,S. (1991) J. Clin. Invest., 87, 133-138.
9. Hagiwara,H., Kozuka,M., Sakaguchi,H., Eguchi,S., Ito,T. and Hirose,S. (1991) J. Cardiovasc. Pharmacol.(in press).
10. Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K.(eds.) (1987) Current Protocols in Molecular Biology, Green Publishing and Wiley-Interscience, New York.
11. Sanger,F., Nicklen,S. and Coulson,A.R. (1977) Proc. Natl. Acad. Sci. U.S.A., 74, 5463-5467.
12. Kozak,M. (1987) Nucleic Acid Res., 15, 8125-8148.
13. Andersson,S., Davis,D.L., Dahlback,H., Jornvall,H. and Russell,D.W. (1989) J. Biol. Chem.,264, 8222-8229.
14. Takayanagi,R., Kitazumi,K., Takasaki,C., Ohnaka,K., Aimoto,S., Ohashi,M. and Nawata,H. (1991) FEBS Lett.(in press).