A Novel Peptide, Vasoactive Intestinal Contractor, of a New (Endothelin) Peptide Family

MOLECULAR CLONING, EXPRESSION, AND BIOLOGICAL ACTIVITY*

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A new peptide family (endothelin (ET)) consisting of three members in mammals appears to be present in mice according to genomic Southern blot analysis. Two ET-related genes were identified by cloning and sequence analysis of a mouse genome. One encoded a peptide identical to porcine and human vasoconstrictor peptide ET, and the other encoded a novel peptide differing from ET in 5 amino acid residues, with 4 cysteines in the same positions as in ET. This novel peptide was synthesized and confirmed to have in vivo pressor activity similar to that of ET. Northern blot analysis, however, indicated the gene of this novel peptide to be expressed only in the intestine, and not in other tissues or cell lines, or endothelial cells. Furthermore, the peptide evoked a strong contractile response in the guinea pig ileum. This peptide may thus be reasonably classified as a gastrointestinal peptide, vasoactive intestinal contractor.

Vascular tone reflects a balance between neural, humoral, autacoidal, and myogenic factors with dilator or constrictor activities. The discovery of acetylcholine-induced, endothelium-dependent vasodilation (1) stimulated intense interest in the role of the endothelium in modulating vascular responsiveness (2–5). From the culture medium of porcine aortic endothelial cells, we isolated a 21-residue peptide, endothelin (ET), a very potent vasoconstrictor which regulates vascular smooth muscle tone (6). A cDNA encoding human ET was cloned and the amino acid sequences of human and porcine ET-related genes were identified by cloning and sequencing both strands. The standard recombinant DNA procedures used are as described (14, 15).

MATERIALS AND METHODS

Isolation and Sequencing of Mouse ET and VIC Genes—A mouse genomic library was constructed by insertion of BALB/c mouse liver genomic DNA partially digested with Sau3AI into the BamHI sites of Charon 30 (kindly provided by N. Takahashi, Nagoya University, Japan). Approximately 2 × 10^6 plaques from the library were screened with a hybridization probe derived from an EcoRI-EcoRI 1.1-kb fragment of human ET cDNA (7). Restriction fragments of the genomic inserts were subcloned into plasmid pUC 18 and sequenced by the dideoxy chain termination method (13). All DNA sequences were confirmed by sequencing both strands. The standard recombinant DNA procedures used are as described (14, 15).

Southern Blot Analysis—Total genomic DNAs were digested with BamHI, electrophoresed in a 0.7% agarose gel, and transferred to nitrocellulose filters by the method of Southern (16). The filters were hybridized at 65 °C in a solution containing 1 M NaCl, 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 0.1% SDS (1:2 Denhardt's solution (1:2 Denhardt's = 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.1% SDS, 50 μg/ml salmon sperm DNA and 6 × 10^6 cpm/ml probe. Fragments of the 32P-labeled probes are shown in Fig. 1A. The filters were washed first at low stringency (1 × SSC (0.15 M NaCl, 0.015 M Na citrate), 0.1% SDS at 40 °C) and then at high stringency (0.1 × SSC, 0.1% SDS at 65 °C).

Synthesis of Mouse VIC—Mouse putative VIC was assembled with an Applied Biosystems model 430A peptide synthesizer according to the primary structure predicted from the nucleotide sequence. The purity of the final product was determined by analytical HPLC and amino acid analysis. The disulfide bond arrangement of synthetic VIC was the same as that of ET, based on HPLC profiles (6, 8).

Assay of In Vivo Pressor Effect in Conscious Rats—Male Wistar rats (350–400 g) were used. After synthetic ET or mouse VIC had been injected intravenously, blood pressure was measured by the tail-cuff method (17).

RESULTS AND DISCUSSION

On using a human ET cDNA fragment (EcoRI–EcoRI, 1.1 kb (7)) for analysis of a Southern blot of a BALB/c mouse genomic DNA at low stringency, a thick band, possibly indicating the mouse ET gene, and several faint bands, possibly genes homologous to ET, were observed (data not shown). The same probe detected 20 hybridization-positive clones from 2 × 10^6 screenings with a BALB/c mouse liver genomic DNA library. These clones were subdivided into two groups, mET and mVIC, by restriction enzyme mapping (Fig. 1A). Fragments of the mET and mVIC probe regions indicated by

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M26497 and M26498.

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2 The abbreviations used are: ET, endothelin; VIC, vasoactive intestinal contractor; EHP, endothelin homologous peptide; kb, kilobase pairs; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography.
Vasoactive Intestinal Contractor Forms an Endothelin Family

Fig. 1. Presence of ET and VIC genes in a mouse genome. A, restriction enzyme mapping of ET and VIC genes. Vertical bars show regions encoding ET and VIC in mET and mVIC, respectively. Open boxes indicate the left arm (EcoRI-BamHI fragment) of the phase vector (Charon 30). Regions of the mET probe (Sau3AI-XbaI, 0.3 kb) and the mVIC probe (PstI-BamHI, 0.4 kb) are indicated by small horizontal lines overlapping ET and VIC, respectively. Each probe has a small portion of the putative intron sequences on either side. H, HindIII; S, SacI; B, BamHI. B, total DNA Southern blotting of mouse ET and VIC genes at low stringency. BALB/c mouse liver DNA digested with BamHI was hybridized with an mET or an mVIC probe (Fig. 1A). An mET probe hybridized with the 19.4-kb band, while an mVIC probe hybridized with the 1.8-kb band. Other bands showed cross-hybridization. The faint band between the 19.4- and 1.8-kb bands shows a gene homologous to the ET and VIC genes. At high stringency, all cross-hybridized bands disappeared. The other BamHI site of the 19.4-kb band must surely be present in the uncloned region of mET in A.

small horizontal lines in Fig. 1A were also hybridized with synthetic oligonucleotides (7) derived from porcine ET. In Southern blotting of BALB/c mouse genomic DNA at high stringency (Fig. 1B), an mET probe hybridized with a 19.4-kb BamHI band and an mVIC probe hybridized with a 1.8-kb BamHI band. This and the mapping data in Fig. 1A indicate mET and mVIC to be located at different genomic loci.

Sequence analysis of mET and mVIC indicated the genes of two putative peptides, ET and VIC, to be present in the mouse genome. Mouse ET was identical to porcine and human ET, while VIC was a novel peptide homologous to ET, demonstrating the possible presence of an ET family in the mouse (Fig. 2). The nucleotide and amino acid sequences of this ET family from several species were compared (Fig. 2). ET and its flanking amino acid residues were completely identical in mouse, pig, and human. But mouse VIC had 3 amino acid residues differing from those of ET, at amino acid positions 9, 11, and 12; that is, Ser, Leu, and Met changed to Asn, Trp, and Leu, respectively. These substitutions were very conservative (from polar to polar and non-polar to non-polar). The positions of the 4 cysteine residues and a dibasic pair of amino acids, Arg-Arg, directly preceding the mature peptides and recognized by processing endopeptidases (21), were also conserved. Trp-Val, possibly recognized by the “ET-converting enzyme” (6), was also conserved.

In Southern blotting of mouse genomic DNA at low stringency (Fig. 1B), a faint band between the 19.4- and 1.8-kb bands showed a gene homologous to the ET and VIC genes. In Southern blotting of rat genomic DNA hybridized with mET and mVIC probes, the hybridization-positive bands differing in size from rat ET (8) suggest that the ET and VIC genes in the mouse may also be present in the rat. Rat ET (8) (designated as rat EHP in Fig. 2) has 6 amino acids differing from those of ET or VIC, the substitutions were less conservative, and the recognition site of the ET-converting enzyme changed from Trp-Val to Trp-Ile. These facts indicate rat ET to possibly be a third member of the ET family, an “ET homologous peptide” (EHP), whose expression has yet to be confirmed (8). Thus, the gene corresponding to the faint band between the 19.4- and 1.8-kb bands in Fig. 1B suggest the presence of EHP in the mouse. Since our discovery of the mouse ET family,3 human ET family genes have been reported,4 although none of them is identical to mouse VIC. We observed5 by Southern blot analysis the presence of ET family genes in Xenopus as well. The presence of the ET family (ET, VIC, and EHP) in vertebrates may thus be a possibility. The amino acid sequences of ET, VIC, and EHP differ mainly in their amino-terminal halves, and their conserved carboxy-terminal halves may be essential for binding to receptors, as observed for the ligand binding site of IL-2 receptors (22, 23).

The structural homology between ET and VIC prompted us to synthesize VIC and examine its pressor activity. Intravenous injection of VIC exerted as characteristically long lasting an in vivo pressor effect in conscious Wistar rats (Fig. 3) as ET (6). VIC (1 nmol/kg) caused a rise of 22 mm Hg (1 mm Hg = 1.333 × 102 pascal, pascal = mg−1 kg s−2) in blood pressure, and the profile of in vivo pressor activities was essentially the same as that for ET (6). Thus, possibly VIC may be a novel vasoconstrictor peptide with a potency nearly equal to that of ET.

To examine the tissue-specific expression of the ET and VIC genes, poly(A)+ RNAs isolated from various mouse tissues, mouse cell lines, and cultured bovine and human endothelial cells were analyzed by Northern blotting (Fig. 4). An mET probe (Fig. 1A) hybridized with a 2.3-kb transcript in bovine and human endothelial cells as expected, but this probe failed to detect any transcripts in other cells or tissues. In contrast, an mVIC probe (Fig. 1A) hybridized with a 1.4-kb transcript in the intestine but not in other tissues, cell lines, or endothelial cells (Fig. 4). It is thus evident that the expression of ET and VIC genes is regulated in a tissue-specific manner.

In consideration of the tissue-specific expressions of the ET and VIC genes, the possibility that VIC may have contractile effect on the intestine was examined. In vitro isotonic contraction activity of VIC in guinea pig ileum was confirmed and calculated as 29.8 ± 6.7% (at 10−6 M VIC, n = 3), this value being the percentage of contraction induced by 10−6 M acetylcholine. Based on the above results, VIC may be classified as a gastrointestinal peptide in consideration of its tissue-specific expression and activity. The biological characterization of VIC has been presented in greater detail (24).

3 N. Ishida, unpublished data.
4 Saida, K., Mitsui, Y., Nishimatsu, S., and Ishida, N. (1988) Scientific Meeting of the Molecular Biology Society of Japan, December 20, 1988, p. 220, Tokyo, Japan.
5 Yanagisawa, M., Inoue, A., Kimura, S., Kasuya, Y., Miyachi, T., Goto, K., and Masaki, T. (1989) Scientific Meeting of Japanese Pharmacological Society, March 26, 1989, p. 142, Kyoto, Japan.
Vasoactive Intestinal Contractor Forms an Endothelin Family

Mouse VIC

1
CTG CGT TTT CCA TCC TCC AAG TCC TGC CAC CGC GAA CAC CTG GAC ATC ATC TGG AAG ACT GCG GG

Mouse ET

1
CTC AGG TCC AAG CCC TCC TCC TCC TCC ATG CAA GAA GAG TGC TAC TCC TCC CAC CGC GAC ATC ATC TGG GAC ACT CCC GA

Porcine ET

1
CTG CGG TCC AAG CCC TCC TCC TCC TCC ATG CAA GAA GAG TGT GTC TAC TCC TGC CAC CGC GAC ATC ATC TGG GAC ACT CCC GA

Human ET

1
CTC CGG TCC AAG CCC TCC TCC TCC TCC ATG CAA GAA GAG TGC TAC TCC TGC CAC CGC GAC ATC ATC TGG GAC ACT CCC GA

Rat EHP

1
CAC CGA CCT CGG CCC TGC AAC TGC TCC TCC TCC TCC ATG CAA GAA GAG TGC TAC TCC TGC CAC CGC GAC ATC ATC TGG GAC ACT CCC GA

FIG. 2. Comparison of primary structures of the ET family. Partial nucleotide and deduced amino acid sequences of the mouse, porcine (6), human (7), and rat (8) ET (designated here as rat “ET homologous peptide” (EHP)) family are compared. Open boxes show regions of the mature peptide (21 amino acid residues). Nucleotides and amino acids differing from those of porcine ET are underlined. The putative introns for mouse and rat genes are typed in small letters. These sequences fit well with the consensus sequence for the 5′ end of the intron (25). Nucleotide and amino acid numbers are indicated above the structures.

FIG. 3. In vivo pressor effects of ET and mouse VIC in a conscious rat. Increasing doses (nanomoles/kg) of ET (●; n = 4) or mouse VIC (■; n = 4) were administered intravenously and blood pressure monitored.

In conclusion, we have demonstrated the existence of a gene for a novel peptide, the VIC, and ET and EHP genes as well, suggesting the existence of a new peptide (endothelin) family. In consideration of the findings that the VIC gene is expressed in mouse intestine but not in endothelial cells, and that VIC induces strong contraction of the guinea pig ileum, VIC may possibly function as a gastrointestinal peptide. The production of a specific antibody against VIC which does not cross-react with ET or EHP is now being carried out to identify VIC-producing cells in the intestine and study the physiological and pathological roles of VIC.

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FIG. 4. Differential distribution of ET and VIC mRNAs by Northern blot analysis. RNA origins: E, bovine aortic endothelial cells; I, mouse intestine. An mET probe (Fig. 1A) hybridized with a 2.3-kb transcript in endothelial cells, but an mVIC probe hybridized with a 1.4-kb transcript in the intestine.
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