Clostridium perfringens Enterotoxin Utilizes Two Structurally Related Membrane Proteins as Functional Receptors in Vivo*

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Human and mouse cDNAs showing homology to the Clostridium perfringens enterotoxin (CPE) receptor gene (CPE-R) from Vero cells (DDBJ/EMBL/GenBank™ accession no. D88492) (Katahira, J., Inoue, N., Horiguchi, Y., Matsuda, M., and Sugimoto, N. (1997) J. Cell Biol. 136, 1239–1247) were cloned. They were classified into two groups, the Vero cell CPE receptor homologues and rat androgen withdrawal apoptosis protein (RVP1; accession no. M74067) homologues, based on the similarities of primary amino acid sequences. L929 cells that were originally insensitive to CPE became sensitive to CPE on their transfection with cDNAs encoding either the CPE receptor or RVP1 homologues, indicating that these gene products are not only structurally similar but also functionally active as receptors for CPE. By binding assay, the human RVP1 homologue showed differences in affinity and capacity of binding from those of the Vero cell CPE receptor. Northern blot analysis showed that mouse homologues of the CPE receptor and RVP1 are expressed abundantly in mouse small intestine. The expression of CPE-R mRNA in the small intestine was restricted to crypt enterocytes, indicating that the CPE receptor is expressed in intestinal epithelial cells. These results are consistent with reports that CPE binds to the small intestinal cells via two different kinds of receptors. High levels of expression of CPE-R and/or RVP1 mRNA were also detected in other organs, including the lungs, liver, and kidneys, but only low levels were expressed in heart and skeletal muscles. These results indicate that CPE uses structurally related cellular proteins as functional receptors in vivo and that organs that have not so far been recognized as CPE-sensitive have the potential to be targets of CPE.

The enterotoxin produced by Clostridium perfringens (CPE) is a simple protein with a molecular weight of ~35,000. Known as a causative agent of diarrhea, this organism (1) elicits fluid accumulation in the intestinal tract by altering the membrane permeability of intestinal epithelial cells (2, 3). Pore formation in the cytoplasmic membrane is now accepted as the underlying mechanism of its effect (4–7). Not only humans, but also various experimental animals have been shown to be sensitive to CPE (8), suggesting that the sensitivity is not restricted to a particular species. Although the natural target of CPE is the intestine, CPE has also been detected in other tissues and organs, including the liver and kidneys, after its intravenous injection into rats and mice (9). In addition, cultured cells of the intestine, liver, and kidneys from various species have been shown to be sensitive to CPE (10–14). Two different receptors with high and low affinity to CPE have been found on the surface of rabbit intestinal epithelial cells (10). Horiguchi et al. (14) showed that Vero cells and Madin-Darby canine kidney cells, both of which are derived from kidneys, express high and low affinity receptors, respectively. Since the cytotoxic action of CPE to target cells requires its binding to specific receptors (14, 15), at least two molecules with different affinities to CPE are considered to exist in various organs of a wide range of species.

Recently, we cloned a cDNA for the CPE receptor (CPE-R) from a CPE-sensitive Vero cell cDNA library (16). The cDNA encodes a highly hydrophobic transmembrane protein of ~22 kDa, the physiological functions of which have not yet been elucidated. The amino acid sequence of the Vero cell CPE receptor showed close similarity to that of the rat androgen withdrawal apoptosis protein RVP1 (17). The Vero cell CPE receptor corresponds to the reported high affinity binding site for CPE. CPE-R was found to be expressed in CPE-sensitive cell lines from different origins; i.e. Vero cells from monkey kidneys, Henle intestine 407 cells from human small intestine, and Hep3B cells from human liver. In contrast to these sensitive cells, CPE-insensitive cells, such as the human erythroleukemia cell line K562 and human lymphoblastoid cell line JY, were found not to express CPE-R, suggesting that the expression of CPE-R is tissue- and/or organ-specific.

In this study, to obtain further insight into the receptors for CPE in vivo, we sought for human and mouse cDNAs similar to the Vero cell CPE-R. Here we report that these gene products can be classified into two groups homologous to the CPE receptor and RVP1, based on their similarities in structure to the Vero cell CPE receptor and RVP1 and differences in their affinities and binding capacity to CPE, and that they both have the ability to confer CPE sensitivity to the insensitive L929 cell line. We also found that the expressions of CPE-R and RVP1 were observed in a wide variety of organs, including the small intestine. These data indicate that two different gene products are expressed in target organs in vivo and are probably recognized as different affinity receptors by CPE.

EXPERIMENTAL PROCEDURES

Identification of Mouse and Human Homologues of CPE-R and RVP1, and Plasmid Construction—Human and mouse genes showing
homology to the Vero cell CPE-R—

**Molecular Cloning of Low and High Affinity CPE Receptors**

3. **Northern Blot Analysis**—The expression of CPE-R and RVP1 in various tissues of mice was examined by Northern blot analysis performed on Mouse Multiple Tissue Northern blot (CLONTECH). An EcoRI-NoI fragment containing the entire coding region as part of the 5'-untranslated region of pMeEmCPE-R or an EcoRI-HincII fragment of pBSmRVP3 containing a part of the coding region and 3'-untranslated region of mouse RVP1 were radiolabeled with [32P]dCTP and used as probes. Hybridization was performed in 5× SSPE (1× SSPE is 180 mM NaCl, 10 mM sodium phosphate buffer (pH 7.7), 1 mM EDTA) at 68 °C for 16 h, washed twice with 1× SSPE at 60 °C, and exposed to an imaging plate for 20 h. The blots were rehybridized with [32P]-labeled human EF-1α cDNA probe (25) to confirm the amounts and integrity of the samples.

4. **In Situ Hybridization**—Two-month-old ddY mice bred in our colony were killed, and the small intestines were rapidly removed, embedded in OCT compound (Tissue Tek II, Miles, Elkhart, IN), frozen in isopentane in dry ice, and sectioned (6 μm) with a cryostat. The sense and antisense cRNA probes for mouse clone 303411 (nucleotides 1–851) were prepared by in vitro transcription using a T4/T7 transcription kit (Boehringer Mannheim) with biotin-16-uridine-5'-triphosphate (Boehringer Mannheim). Sections on slides were hybridized for 12 h at 50 °C to the biotin-labeled probes in aliquots of 100 μl of hybridization buffer (50% formamide, 10 mM Tris-HCl (pH 7.6), 1× Denhardt's solution, 10% dextran sulfate, 600 mM NaCl, 1 mM EDTA) containing 200 μg/ml yeast tRNA. The slides were washed with 2× SSC (1× SSC contains 0.15 M NaCl and 0.015 M Tris, pH 7.5) and 0.1% SDS at 55 °C and then with 10 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA at 37 °C. Subsequently the sections were treated with 10 μg/ml RNase A, washed with 2× SSC, then with 0.2× SSC at 50 °C, and then incubated with alkaline phosphatase-conjugated streptavidin (Oncogene Science, Uniondale, NY). They were then washed with 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and color was developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Promega, Madison, WI).

**Flow Cytometric Analysis, [125I]-CPE Binding Assay, and Cytotoxicity Assay**—Flow cytometric analysis with a biotinylated CPE C-terminal fragment (H254-PEER) was performed as described previously (16). Briefly, L929/pTi18 cells were transfected with 10 μg of either pMEPyor118sf, pMEmCPE-R, pMeEmCPE-R, or pMbRVP1. After 48 h, the cells were harvested and resuspended in 100 μl of phosphate-buffered saline (PBS) containing 1% BSA (PBS-BSA). Then the cells were treated with biotinylated H254-PEER (10 μg/ml), followed by phycoerythrin-conjugated streptavidin (20 μg/ml, Biomedica Corp., Foster City, CA). They were then washed with PBS-BSA, and their fluorescence was examined in a FACScan (Bekton Dickinson, Mountain View, CA).

**RESULTS**

**Identification and Sequence Analysis of cDNAs Showing Homology to Vero cell CPE-R**—I.M.A.G.E. cDNA clones 25804 from an infant human brain library, which showed the highest homology to Vero cell CPE-R and clone 303411 from a fetal mouse kidney, were sequenced, and the deduced amino acid sequences were compared with that of the Vero cell CPE receptor. They contained small ORFs encoding 209 (clone 25804) and 210 (clone 303411) amino acids, respectively. The amino acid sequences of these clones were highly homologous (clone 25804, 99.0% homology; clone 303411, 83.8% homology) to that of the Vero cell CPE receptor (Fig. 1A, vCPE-R) and had four highly hydrophobic domains with potentials to form transmembrane domains (Fig. 1B). Thus we designated these genes as...
hCPE-R (clone 25804; accession no. AB000712) and mCPE-R (clone 303411; accession no. AB000713). The ORF of clone 214937 was truncated, so the complete ORF was deduced from the nucleotide sequences of the clone 214937 and the 3'-RACE product (see "Experimental Procedures"). It consisted of 220 amino acids and also contained four putative transmembrane

**FIG. 1.** Structural relationships of CPE-receptors and RVP1. A, comparison of amino acid sequences of CPE-R and RVP1 gene products. Multiple alignment of the amino acid sequences of the CPE receptors of Vero cell (vCPE-R, accession no. D88492), human (hCPE-R, I.M.A.G.E. clone 25804, accession no. AB000712), mouse (mCPE-R, I.M.A.G.E. clone 303411, accession no. AB000713), and RVP1 from rat (rRVP1, accession no. M74067) and human (hRVP1, I.M.A.G.E. clone 214937, accession no. AB000714) cells was done by the CLUSTAL method (24). Identical amino acids are shown by white letters in black boxes and conservative replacements are shadowed. B, hydrophobicity profiles of the CPE receptor from Vero cells (a), human (b), and mouse (c) cells and RVP1 from human (d) cells. The hydrophobicity index was determined by the Kyte and Doolittle (29) algorithm.
domains (Fig. 1B). The amino acid sequence of this ORF showed higher homology to that of the rat RVP1 (89.4% identity, 97.7% similarity) than to that of the human CPE receptor (69.9% identity, 96.7% similarity) or the Vero cell CPE receptor (70.9% identity, 96.6% similarity). We, therefore, named this gene encoding the human homologue of RVP1 as hRVP1. The amino acid sequence of the mouse CPE-R gene product showed higher homology to that of the Vero cell CPE receptor than to that of the rat RVP1 (66.8% identity, 94.6% similarity) or human RVP1 (66.0% identity, 94.3% similarity). Taken together, we concluded that the EST clones which showed similarity to the Vero cell CPE-R gene could be classified into RVP1 and CPE-R homologues and that these two molecules are not expressed from the same locus by alternative splicing but are encoded on different loci.

The Homologous Genes Encoding the Functional CPE Receptor—We tested whether these gene products actually act as functional receptors for CPE. The human and mouse CPE receptors and the human RVP1 were each transiently expressed in L929pyT18 cells, and flow cytometric analysis using the biotinylated H10PER probe was performed as described previously (16). A peak showing increased fluorescent intensity was observed when the human and mouse CPE receptor homologues (Fig. 2, B and C, thick lines) as well as the Vero cell CPE-R cDNA (Fig. 2A, thick line) were expressed. When human RVP1 was expressed in L929pyT18 cells, cells brighter than the background appeared, but they did not form any separated peaks due to their relatively low fluorescent intensity (Fig. 2D, thick line). This tendency, confirmed by three independent experiments (data not shown), could be due to both its lower affinity and fewer expression on these cells as shown below. When the pMEPyori18sf vector was introduced into the cells, no increase of the fluorescent intensity was observed (Fig. 2, A–D, thin lines).

We then tested the CPE sensitivities of L929pyT18 cells transiently expressing the CPE receptor homologues and hu-
the human EF-1 α panel (9 portion of the ORF and 3′-untranslated region and a part of the 5′-noncoding region of mouse CPE-R (upper panel)). The blot was rehybridized with 32P-labeled mouse RVP1 probe containing the 3′-portion of the ORF and 3′-untranslated region of mouse RVP1 cDNA (middle panel; accession no. AB000715). The blot was rehybridized with 32P-labeled human EF-1α probe (lower panel) to confirm the amounts of the samples. The positions of 28S and 18S ribosomal RNAs are indicated on the left.

The expression of CPE-R in other mouse tissues was also tested. Two kinds of transcripts of different sizes were expressed (Fig. 5B, upper panel). Kidneys expressed a transcript of about 1.8 kb abundantly. This 1.8-kb transcript was also detected in heart and skeletal muscle, although at lower levels. In liver, a shorter transcript of about 1.3 kb was expressed in place of the 1.8-kb transcript. In lungs, both the 1.8- and 1.3-kb transcripts were detected. The presence of transcripts of different sizes is consistent with the results of a database search except for the pattern in mouse liver, in which the 1.3-kb transcript was the major one found. The cDNA fragment corresponding to a part of the 3′-noncoding region of mouse RVP1 hybridized with the 1.3-kb transcript, but not with the 1.8-kb transcript in stringent washing conditions, indicating that RVP1 is the major transcript in place of CPE-R in mouse liver (Fig. 5B, middle panel). The expression of RVP1 was also detected in intestine but not in L929 cells (Fig. 5A, middle panel). The lack of the expression of both CPE-R and RVP1 in L929 cells coincides well with their lack of the sensitivity to CPE. Brain, spleen, heart, skeletal muscle, and kidneys (Fig. 5B, middle panel) also expressed RVP1, but at much lower levels than in intestine, liver, and lungs. No expression of 1.8-kb CPE-R mRNA was detected in brain or spleen.

CPE-R mRNA in Small Bowel Crypts of an Adult Mouse—For determination of the histological localization of CPE-R mRNA in mouse small intestine, in situ hybridization was performed. In the jejunum of adult mice, intense positive staining was observed in the cryptic cells when biotin-labeled antisense-CPE-R cRNA probe was used (Fig. 6A). Enterocytes in the lower part of the villi exhibited either faint or, in most cases, no positive signals for CPE-R mRNA. The upper half of the villi showed no positive signals. Cells from the upper to lower parts of the villi and cryptic cells showed no positive signal with biotin-labeled sense-CPE-R cRNA as a probe (Fig. 6B). The probe used in this experiment contained the coding region of the CPE-R gene, and thus could hybridize with both CPE-R and RVP1 mRNAs (see Fig. 5, A and B, upper panels). However, because it hybridized much more stably with CPE-R, the majority of positive signals indicated the presence of CPE-R mRNA.

DISCUSSION

Two Different Kinds of Molecules Act as Receptors for CPE—In the present study we found that the cellular proteins...
that function as receptor for CPE in vivo could be classified into two different groups of the CPE receptor and RVP1 types. These receptors were very similar in structure but had different affinities to CPE. Also, we found that the cell lines expressing human RVP1 showed lower binding capacity to CPE than those expressing human CPE receptor. McDonel and McClain (15) reported that high and low affinity receptors are both expressed by a single cell line (Vero cells). However, we previously observed only a 1.8-kb CPE transcript in these cells (16). Possibly Vero cells express RVP1 as a low affinity receptor, but its hybridization to the CPE probe was too unstable to detect its expression (note that the signal intensity of RVP1 transcript obtained with the CPE-R probe was much weaker than that obtained with the RVP1 probe; see Fig. 5B). Northern blot analysis of the expression of RVP1 mRNA in Vero cells using RVP1 probe will solve this problem. Further examination of whether CPE-R and RVP1 are expressed concomitantly in the same cell population or separately in specific cell types will also provide a clue for understanding the physiological functions of these two proteins.

We detected the expressions of CPE-R and RVP1 in liver. Previously we demonstrated that the CPE-R transcript was expressed in CPE-sensitive Hep3B cells, which are derived from human liver (16). Mouse liver cells in primary culture were CPE-sensitive but exhibited lower affinity to CPE than did Vero cells (11). Since we showed that mouse liver expresses the RVP1 transcript abundantly, it is probably recognized as the target molecule for CPE. The expression of CPE-R in human liver was confirmed by the database search shown in Table I and Northern blot analysis of Hep3B cells, so the possibility that some portion of human hepatocytes may express RVP1 under particular conditions cannot be excluded.

We observed the expression of CPE-R in lungs, heart, and skeletal muscles as well as in intestine, liver, and kidneys. The former organs have not so far been thought to be sensitive to CPE in vivo. Cell lines derived from rat skeletal muscle (L-6 cells) and rat smooth muscle (A-10 cells) were CPE-insensitive. Moreover, we found that a mouse myoblastic cell line C2C12 did not express CPE-R mRNA or showed any morphological alterations by the addition of CPE to the culture medium; even when these cells were induced to differentiate into multinucleate myotubes, they did not show CPE sensitivity (data not shown). We think that the reported insensitivity of the cultured myogenic cell lines may indicate that they do not express sufficient amounts of CPE-R or RVP1 to be sensitive to CPE. It is possible that some accessory molecules that are essential for CPE-induced cytotoxicity are not present in myogenic cells, resulting in their insensitivity. However, this possibility is unlikely, since a C2C12 cell line stably expressing the Vero cell CPE-R became sensitive to CPE (data not shown). The mechanisms regulating expression of the CPE receptor and RVP1 in specific cells in vivo remain to be elucidated.

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