

# Molecular Cloning and Functional Characterization of the Receptor for *Clostridium perfringens* Enterotoxin

Jun Katahira,\* Norimitsu Inoue,‡ Yasuhiko Horiguchi,\* Morihiko Matsuda,\* and Nakaba Sugimoto\*

\*Department of Bacterial Toxicology and ‡Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, Osaka 565, Japan

**Abstract.** A cDNA encoding the *Clostridium perfringens* enterotoxin receptor gene (*CPE-R*) was cloned from an expression library of enterotoxin-sensitive Vero cells. The nucleotide sequence of *CPE-R* showed that the enterotoxin receptor consists of 209 amino acids with a calculated molecular mass of 22,029 D. This receptor is highly hydrophobic, contains four putative transmembrane segments, and has significant similarity to the rat androgen withdrawal apoptosis protein RVP1 and the mouse oligodendrocyte specific protein, the functions of which are unknown. The expression of *CPE-R* was detected in the enterotoxin-sensitive Vero,

Hep3B, and Intestine 407 cell lines, but not in the enterotoxin-insensitive K562 and JY cell lines. The *CPE-R* gene product expressed in enterotoxin-resistant L929 cells bound to enterotoxin specifically and directly and with high affinity and rendered the cells sensitive to the toxin, indicating that the cloned receptor is functional. Results showed that enterotoxin could not assemble into a complex with a defined structure unless it interacted with the receptor. From these results, it is proposed that the enterotoxin receptor is required for both target cell recognition and poreformation in the cell membrane.

**C**LOSTRIDIUM *perfringens* enterotoxin (CPE)<sup>1</sup>, which consists of a single polypeptide chain and has a molecular weight of ~35,000, is the causative agent of symptoms associated with *C. perfringens* food poisoning in man (McClane et al., 1988a). CPE produced in the intestinal tract during sporulation injures intestinal epithelial cells and causes fluid accumulation in the intestinal cavity, resulting in diarrhea (Stark and Duncan, 1971). Morphological changes such as bleb balloon formation or complete destruction of intestinal epithelial cells induced by CPE has been observed in rat and rabbit models (McDonel and Duncan, 1975; McDonel et al., 1978). Similar cytotoxic effects have also been shown in cultured mammalian cells (Matsuda and Sugimoto, 1979; McClane and McDonel, 1979; McDonel, 1980; Tolleshaug et al., 1982). Several lines of evidence suggest that CPE increases membrane permeability by forming small pores and induces the release of intracellular molecules from sensitive cells (Matsuda and Sugimoto, 1979; McClane and McDonel, 1981; Sugimoto et al., 1985, 1988; Matsuda et al., 1986). The con-

sequent loss of osmotic equilibrium is considered to lead to membrane destruction, resulting in morphological alterations and finally cell death.

The sensitivities of different cell types to CPE vary. Rabbit small intestinal epithelial cells (McDonel, 1980), rat liver cells (Tolleshaug et al., 1982), the monkey kidney cell line, Vero cells (McClane and McDonel, 1979), and HeLa cells (Matsuda and Sugimoto, 1979) have been shown to be sensitive to CPE. On the contrary, several types of cells such as the mouse fibroblast cell line L929 (Horiguchi et al., 1985) were found to show resistance to CPE. The sensitivity of cells to CPE is thought to depend on the presence of a specific receptor(s) for CPE on the cell surface (McDonel and McClane, 1979; Horiguchi et al., 1985). Pretreatment of CPE-sensitive cells with neuraminidase or addition of ganglioside, methyl  $\beta$ -galactoside, methyl  $\alpha$ -mannose, or N-acetylglucosamine to the medium did not affect the binding ability of CPE, whereas extensive digestion of the sensitive cells with pronase did (McDonel, 1980; Wnek and McClane, 1986; Tolleshaug et al., 1982; McClane et al., 1988b). These findings indicate that CPE binds to a specific protein receptor(s) on the surface of sensitive cells.

Cellular proteins of 50–60 kD derived from sensitive cells were reported to have affinity to CPE (Wnek and McClane, 1983; Sugii and Horiguchi, 1988). However, it is unknown whether these proteins act as a functional membrane receptor(s) for CPE. Wieckowski et al. (1994) reported that after its initial binding, CPE aggregates with

Please address all correspondence to Jun Katahira, Department of Bacterial Toxicology, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565, Japan. Tel.: 81-6-879-8285; Fax.: 81-6-879-8283; E-mail: katahira@biken.osaka-u.ac.jp

1. Abbreviations used in this paper: CPE, *Clostridium perfringens* enterotoxin; *CPE-R*, *CPE*-receptor gene; H<sub>10</sub>PER, histidine-tagged *CPE* COOH-terminal fragment; PE, phycoerythrin.

several cellular proteins and forms a large hydrophobic complex with a molecular mass of 160 kD. By a simple calculation they concluded that the complex contained a 70-kD protein besides the 50-kD CPE binding substance and CPE (35 kD) and assumed that this complex contributed to pore formation in the membrane of target cells. But the composition and function of the CPE-induced complex can not be determined until the functional CPE receptor is identified.

To understand the molecular mechanism of the action of CPE, we isolated the CPE-receptor gene (*CPE-R*) and examined the function of the gene product. Here we report the identification and characterization of a functional CPE receptor.

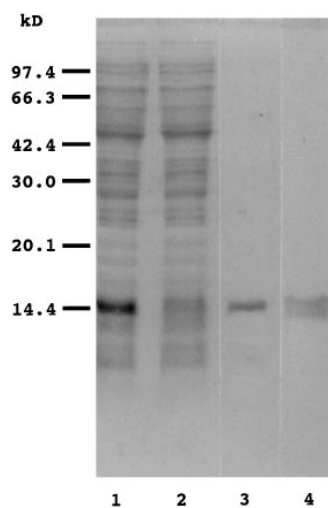
## Materials and Methods

### Plasmid Construction

Genomic DNA was isolated from *C. perfringens* strain NCTC8239 (a gift from Dr. T. Asao, Osaka Prefectural Institute of Public Health, Osaka, Japan) by the method described by Marmur (1961). Approximately 10 ng of the genomic DNA was subjected to PCR using oligonucleotides 5'-CCG-CTCGAGAGATGTGTTTTAACAGTTCCATCTAC-3' (primer-S; the underline indicates XhoI site) and 5'-GGAAGATCTTAAAATTTT-TGAAATAATATTGAATAAGGG-3' (primer-A; the underline indicates BglII site) as sense and antisense primers to amplify the DNA fragment corresponding to amino acid residues 184–319 of CPE (Czeczulin et al., 1993). The amplified DNA fragment was digested with XhoI and BglII and then cloned into the XhoI-BamHI treated pET16b vector (Novagen Inc., Madison, WI) to fuse the CPE fragment to the down stream of the tag sequence with 10 histidine residues (Fig. 1A). The resulting plasmid vector was designated as pETH<sub>10</sub>PER.

For constructing pCMVβ-GAL, a HindIII-PstI fragment containing *Escherichia coli* β-galactosidase gene was isolated from pSGβ-galactosidase (Promega Biotech., Madison, WI) and was subcloned into the same site of pCDM8 (Invitrogen Corp., San Diego, CA).

For sequencing, the clone 706 encoding *CPE-R* was isolated by XhoI digestion followed by treatment with T4 DNA polymerase, and the fragment obtained was introduced into the EcoRV site of pBluescript SK(-) (Stratagene Cloning Systems, La Jolla, CA). Two clones containing the *CPE-R* gene in opposite orientations were obtained and named pBS70608 and pBS70614. Nested deletion mutants of these clones were prepared us-



**Figure 1.** Expression and purification of a histidine-tagged *Clostridium perfringens* enterotoxin COOH-terminal fragment (H<sub>10</sub>PER). H<sub>10</sub>PER was expressed in *E. coli* BL21 (DE3) and purified as described in Materials and Methods. A total cell lysate (lane 1), flow through fraction (lane 2), and purified H<sub>10</sub>PER (lane 3) were separated on SDS-15% polyacrylamide gel and stained with Coomassie brilliant blue. Purified H<sub>10</sub>PER was transferred to a PVDF membrane, and Western blot analysis was carried out using rabbit polyclonal anti-CPE antibody, followed by alkaline phosphatase-conjugated anti-rabbit IgG (lane 4). The positions of molecular weight standards are indicated on the left in kD.

ing a double-stranded Nested Deletion Kit (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's manual.

The CPE receptor cDNA was introduced into pMeneo vector (Watanabe et al., 1996), and the resulting plasmid (pMeneo-CPE-R) was used to establish L929 cell lines stably expressing *CPE-R*.

For construction of a COOH-terminal FLAG peptide tagged CPE receptor expression plasmid, *CPE-R* in pBS70614 was amplified by PCR using the oligonucleotides 5'-GGGTCGACGCCTCCATGGGGCTACAGG-3' (the underline indicates the SalI site) and 5'-GGTCCGACACG-TAGTTGCTGGCAGCAG-3' (the underline indicates the NruI site) as forward and back primers. The amplified fragment was treated with T4 DNA polymerase followed by T4 polynucleotide kinase and cloned into the EcoRV site of the pBluescript SK(-). The XhoI-FseI site of this plasmid was replaced with the fragment of the corresponding site (encoding NH<sub>2</sub>-terminal portion of native CPE receptor) of pBS70614 to generate p706NruI. The XhoI-NruI fragment of p706NruI was then isolated and recloned into the same site of pMEEB (Watanabe et al., 1996) into which NruI site, FLAG sequence, and stop codon (TCGCGAGACTACAAG-GACGACGATGACAAGTAA; the underline indicates NruI site) was introduced. The resulting plasmid was named pMEEB-CPE-R-FLAG.

Plasmids pS7neo (Takahashi et al., 1996) was a gift from Dr. M. Takahashi (Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University). The construction of pMEPyori18Sf(-) is described elsewhere (Ohishi et al., 1996). pMEPyoriLuc was constructed as described previously (Takahashi et al., 1996).

### Expression of the CPE COOH-terminal Fragment in *Escherichia coli*

pETH<sub>10</sub>PER was introduced into the *E. coli* BL21 (DE3) strain, and expression of the CPE COOH-terminal fragment was induced by 1 mM isopropyl β-D-thiogalactopyranoside (Wako Pure Chemical Industry, Osaka, Japan). The *E. coli* cells were harvested, resuspended in buffer A (10 mM Tris-HCl, pH8.0, 400 mM NaCl, 5 mM MgCl<sub>2</sub>, 10% glycerol, 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride, 1 mM β-mercaptoethanol), and then disrupted by sonication. After removal of cell debris by centrifugation, the cell lysate was applied to a Ni<sup>2+</sup> column (HisBind Resin; Novagen Inc.), and the histidine-tagged COOH-terminal fragment of CPE (H<sub>10</sub>PER) was eluted in a 0–1 M imidazole gradient in buffer A. The purified 15-kD fragment (Fig. 1) was biotinylated with sulfo-NHS-LC biotin (Pierce, Rockford, IL) and used as a probe for flow cytometric analysis.

### Flow Cytometric Analysis

Expression of the CPE receptor was examined by flow cytometric analysis after treatment of cells with biotinylated H<sub>10</sub>PER (0.01 mg/ml) followed by phycoerythrin (PE)-conjugated streptavidin (0.02 mg/ml; Biomedica Corp., Foster City, CA). The fluorescence intensity of the cells was examined in a FACS<sup>®</sup>can (Becton Dickinson, Mountain View, CA).

### Cell Culture and Establishment of Stable Cell Lines

Cells were cultured in DMEM supplemented with 10% FCS at 37°C under 5% CO<sub>2</sub> in air. Clonal L929 cell lines stably expressing the polyoma large T antigen were established by electroporation of linearized plasmid pS7neo, followed by G418 (Geneticin; GIBCO BRL, Gaithersburg, MD) selection. Of 20 cell lines tested, the L929 cell line, which exhibited the highest β-galactosidase and firefly luciferase activity of the transfected pCMVβGAL and pMEPyoriLuc, respectively, was selected. This cell line was named L929pyT18 and used as the host cell line for cDNA library screening. L929 cell lines expressing *CPE-R* and its FLAG peptide-tagged version (CPE-R-FLAG) were established in the same manner, except that pMeneo-CPE-R and pMEEB-CPE-R-FLAG were introduced by electroporation followed by G418 or hygromycin (Wako Pure Chemical Industry) selection. The clonal cell lines expressing CPE receptor and FLAG-tagged CPE receptor were identified by flow cytometric analysis and were designated as 706Neo and 706FLAG, respectively.

### cDNA Libraries

A cDNA library was constructed by the method of Gubler and Hoffmann (1983). An exponentially growing Vero cell culture was used as an RNA source. Total RNA was isolated by cesium trifluoroacetic acid isopycnic centrifugation. PolyA<sup>+</sup> RNA was purified by the spinning of two successive oligo-dT cellulose columns (Pharmacia Biotech). PolyA<sup>+</sup> RNA was

reverse transcribed by Superscript reverse transcriptase II (GIBCO BRL) at 45°C and then converted to double-stranded cDNA with the aid of RNase H, *E. coli* DNA polymerase, and *E. coli* DNA ligase (Takara Shuzo Co., Shiga, Japan). After treatment with T4 DNA polymerase (Toyobo Inc., Osaka, Japan) followed by BstXI linker (Invitrogen Corp.) ligation, cDNAs were size selected and inserted into BstXI treated pMEPyori18Sf(-) vector to construct a cDNA library for eukaryotic expression.

### Expression Cloning

The cDNA library was introduced into the L929pyT18 cell line by electroporation and cultured for 2 d before subsequent analysis. Expression of the CPE receptor was examined by flow cytometric analysis as described. Positive cells (the 0.1% most fluorescent of the transfected cells) were initially sorted by FACS<sup>®</sup>Vantage (Becton Dickinson) and pooled, and the transfected plasmids were recovered by the method of Hirt (1967). The rescued plasmids were reintroduced into *E. coli* strain MC1061, amplified, and then used for subsequent screening. This screening was repeated three times. After the third screening, the rescued plasmid clones were divided into 24 pools of 24 plasmids each, and the positive pools were identified by flow cytometric analysis. Finally, positive clones were identified by separating positive pools into single clones.

### DNA Sequencing and Sequence Analysis

The nucleotide sequences of both strands of the cloned gene were determined by the dideoxy chain termination method using Thermo Sequenase (Amersham International, Amersham, UK) and a DNA sequencer (model 373A; Applied Biosystems, Foster City, CA). Sequence comparisons were made using the BLAST search program (Altschul et al., 1990) on an nr-nt data base. Multiple alignment was done by the CLUSTAL method (Higgins and Sharp, 1989).

### Northern Blot Analysis

Total RNA was extracted from various cell lines by guanidinium thiocyanate-CsTFA isopycnic centrifugation using a QuickPrep Total RNA Extraction Kit (Pharmacia Biotech). Samples of 15 µg of total RNAs were subjected to agarose/formaldehyde gel electrophoresis. Northern blot analysis was performed by the method of Sambrook et al. (1989). The 1.7-kb XhoI fragment of clone 706, containing the entire coding sequence and 5'- and 3'-untranslated regions, was radiolabeled by the random priming method (Megaprime DNA labeling system; Amersham Intl.) and used as a probe. The blot was rehybridized with an elongation factor-1 α probe (Uetsuki et al., 1989) to determine the amount of RNA applied. The blots were exposed to an imaging plate for 18 h and analyzed with a Bioimaging Analyzer (BAS1500; Fuji Film Co., Tokyo, Japan).

### Antibodies

Mouse monoclonal anti-FLAG peptide antibody M2 and M2-conjugated Sepharose were purchased from Eastman Kodak Co. (New Haven, CT). Alkaline phosphatase-conjugated goat anti-mouse IgG and streptavidin were from Organon Teknika (Turnhout, Belgium) and Oncogene Science Inc. (Uniondale, NY), respectively. Rabbit polyclonal antibody was raised against CPE toxoid and purified by protein A-Sepharose chromatography (Senda et al., 1995). Biotinylation of anti-CPE antibody was done as described.

### Purification of CPE, Cytotoxicity Assay, and Binding Assay

CPE was purified by the method of Sakaguchi et al. (1973). The cytotoxic effect of CPE on cells was determined by examination of morphological alterations. CPE was radio-iodinated as described previously (Horiguchi et al., 1985). The ability of CPE binding to Neo706 cells ( $0.4 \times 10^6$  cells/assay) expressing *CPE-R* was measured by the method of Horiguchi et al. (1985), except that the binding reaction was done in PBS(-). Scatchard analysis was performed by the SP123 program (Ikeda et al., 1991).

### Ligand Overlay Assay

Binding of CPE to the *CPE-R* gene product in vitro was examined by ligand overlay assay (Manser et al., 1992). Cell lysates (200 µg protein/lane) of L929 and 706FLAG cells were subjected to SDS-15% polyacryl-

amide gel electrophoresis and then transferred to nitrocellulose membranes. After a denaturation and renaturation cycle (Manser et al., 1994), the blots were soaked in PBS-1% BSA containing <sup>125</sup>I-labeled CPE (5 nM) with or without 100-fold molar excess of cold CPE. The membranes were then washed and exposed to an imaging plate, and the radioactive band was located with a Bioimaging Analyzer.

### Immunoprecipitation

706FLAG cells ( $1 \times 10^6$ ) were labeled for 8 h with 50 µCi/ml of [<sup>35</sup>S]methionine. Cells were harvested after brief trypsinization and suspended in 1 ml of DME-10% FCS. 150 pM of purified CPE or H<sub>10</sub>PER was then added to the cell suspension. After incubating at 4° or 37°C for 30 min, the cells were pelleted and lysed with 400 µl of PBS containing 0.5% NP-40 (PBS-N), and the mixture was centrifuged at 12,000 g for 15 min to remove cell debris. The lysate was mixed with 5 µl of anti-FLAG antibody-conjugated Sepharose which had been blocked with PBS-N containing 5% skim milk. The mixtures were incubated at 4°C for 3 h with mild agitation, and the beads were washed three times with PBS-N at 4°C. The precipitated proteins were boiled in SDS-PAGE sample buffer containing 5% β-mercaptoethanol for 3 min and loaded onto SDS-12% polyacrylamide gel. Radioactive bands were visualized with a Bioimaging analyzer.

In immunological studies, L929 and 706FLAG cells were harvested and treated with CPE or H<sub>10</sub>PER at 37°C. Cell lysates were prepared and immunoprecipitated as described. The precipitated proteins were separated by electrophoresis on SDS-15% polyacrylamide gel or SDS-2–15% polyacrylamide gradient gel (Multi Gel 2/15; Daiichi Pure Chemicals Co., Tokyo, Japan) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were probed with biotinylated anti-FLAG or anti-CPE antibodies and then treated with alkaline phosphatase-conjugated streptavidin; and color was developed with NBT/BCIP.

## Results

### Detection of the CPE-R Expressed on the Surface of CPE-sensitive Cell Lines by Flow Cytometric Analysis with a Recombinant CPE COOH-terminal Fragment

We attempted to identify a putative *CPE-R* by expression cloning. For this, we used flow cytometric analysis with a biotinylated CPE COOH-terminal fragment peptide as a probe, since intact CPE might kill cells expressing the CPE receptor, making it difficult to isolate cells with the *CPE-R*. On the basis of reports that the most COOH-terminal part of CPE functions as the receptor binding domain (Horiguchi et al., 1986, 1987; Hanna et al., 1991, 1992), we used amino acid residues 184–319 of CPE as a probe and constructed a CPE COOH-terminal peptide (H<sub>10</sub>PER) expression system in which the COOH-terminal fragment was fused to 10 consecutive histidine residues followed by linker peptides to facilitate its purification. H<sub>10</sub>PER was expressed in *E. coli* and was purified by Ni<sup>2+</sup> chelating column chromatography. As expected, a single polypeptide of ~15 kD was purified to almost homogeneity (Fig. 1, lane 3). The purified protein was recognized by rabbit anti-CPE serum (Fig. 1, lane 4) and protected Vero cells from CPE-induced cell lysis (data not shown).

The purified H<sub>10</sub>PER was biotinylated, and its cell surface binding ability was examined by flow cytometric analysis. When Vero cells were treated with biotinylated H<sub>10</sub>PER followed by phycoerythrin-conjugated streptavidin, the intensity of fluorescence increased markedly, whereas no increase was observed when biotinylated H<sub>10</sub>PER was omitted from the reaction (Fig. 2 A). The intensity of fluorescence decreased after pretreatment of the cells with unlabeled H<sub>10</sub>PER (Fig. 2 A, thin line). When the human hepatoma cell line Hep3B (Fig. 2 B) or the human intesti-

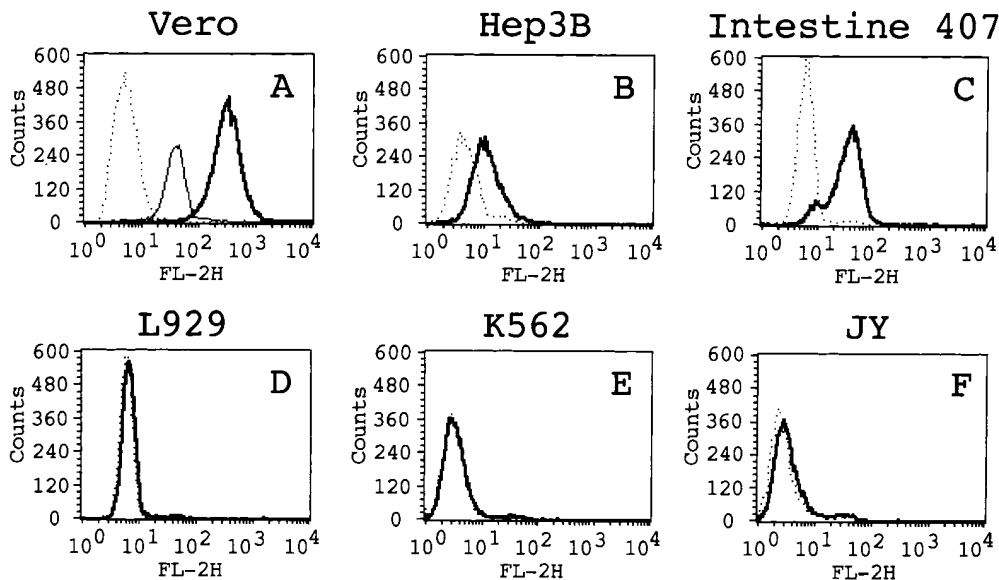


Figure 2. Detection of the CPE receptor by a flow cytometric analysis. Vero (A), Hep3B (B), Henle Intestine 407 (C), L929 (D), K562 (E), and JY (F) cells were treated with biotinylated H<sub>10</sub>PER (thick lines) or buffer alone (dotted lines) followed by PE-conjugated streptavidin. For (A), cells were preincubated with unlabeled H<sub>10</sub>PER as a competitor (thin line). The plots show cell numbers on the ordinate and relative fluorescences on a log scale on the abscissa.

nal epithelial cell line Henle Intestine 407 (Fig. 2 C), which are sensitive to CPE (data not shown), was used as target cells in this analysis, the intensity of fluorescence increased, although the levels of intensity were somewhat lower than those of Vero cells. The binding of biotinylated H<sub>10</sub>PER to L929 cells, which are known to be CPE-insensitive (Horiguchi et al., 1985), did not increase the fluorescence intensity (Fig. 2 D). H<sub>10</sub>PER did not bind to several cell lines that are resistant to CPE-induced cell lysis (data not shown), including K562 cells (Fig. 2 E) and JY cells (Fig. 2 F). These results show that H<sub>10</sub>PER specifically bound to the cell surface receptors on Vero, Hep3B, and Intestine 407 cells.

### CPE-R Encodes a Highly Hydrophobic Membrane Protein

To identify CPE-R, we performed expression cloning. A cDNA library from the CPE-sensitive Vero cell line was introduced into 10<sup>7</sup> L929pyT18 cells that stably express polyoma virus large T antigen (data not shown; see Materials and Methods) and do not bind CPE (Fig. 3 A, thick line). 48 h after transfection, expression of CPE receptor was monitored by flow cytometric analysis, as described, and bright cells were sorted. By this screening, the brightest 1,000 cells among 10<sup>7</sup> transfected cells were initially selected. After three rounds of screening (Fig. 3 A, thin line), several positive clones were obtained. Clone 706 contained a fragment of ~1.7-kb DNA. Other positive clones were screened by Southern blot analysis using the 1.7-kb XhoI fragment from clone 706 as a probe and found to encode the same gene (data not shown).

Sequence analysis revealed that the longest open reading frame of this clone was 630 bp and that it contained a polyadenylation signal followed by a polyA tail at its 3' end (Fig. 3 B), suggesting that the gene is functionally expressed in Vero cells. Two potential AUG initiation codons are present at nucleotide positions 137–139 and 146–148 in the same reading frame. Since the neighboring sequence of the first AUG is consistent with the consensus sequence proposed by Kozak (1987), this was assigned as the initiation codon of CPE-R. Thus, CPE-R encodes a protein of

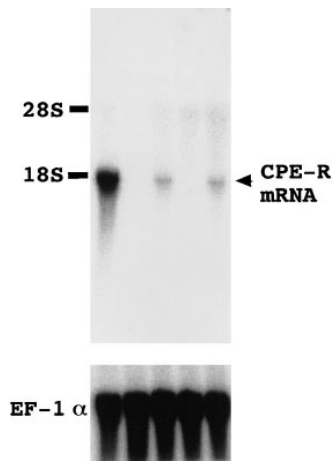
209-amino acid residues with a calculated molecular mass of 22,029 D. The protein consists mainly of hydrophobic amino acids and contains four putative transmembrane domains (Fig. 3 C) and several potential phosphorylation sites in the stretches of hydrophilic residues (Fig. 3 B). A search of data bases indicated that the amino acid sequence of the CPE-R product showed homology to proteins designated as the androgen withdrawal apoptosis protein RVP1 (67.8% homology, 95.6% similarity) and the oligodendrocyte-specific protein (28.7% homology, 63.8% similarity; Fig. 3 D). The latter half of the nucleotide sequence of clone 706, however, showed considerable difference from the RVP1 gene and contained a termination codon at nucleotide position 764–766. Accordingly, clone 706 lacked about one fifth of the COOH-terminal amino acid sequence of RVP1.

We examined the expression of CPE-R in various cell lines by Northern blot analysis with the clone 706 probe. Of five primate cell lines tested, Vero cells expressed the highest level of a transcript of about 1.8 kb. Its expression was also observed in CPE-sensitive Hep3B and Intestine 407 cell lines but at considerably lower levels than in Vero cells. No expression of CPE-R was detectable in the CPE-insensitive K562 and JY cell lines (Fig. 4, upper panel). The levels of expression of human elongation factor 1 $\alpha$  gene in these cell lines were almost the same (Fig. 4, lower panel). The levels of expression of CPE-R correlated well with the CPE-binding abilities of these cell lines (Fig. 2), indicating that clone 706 encodes a functional CPE receptor protein.

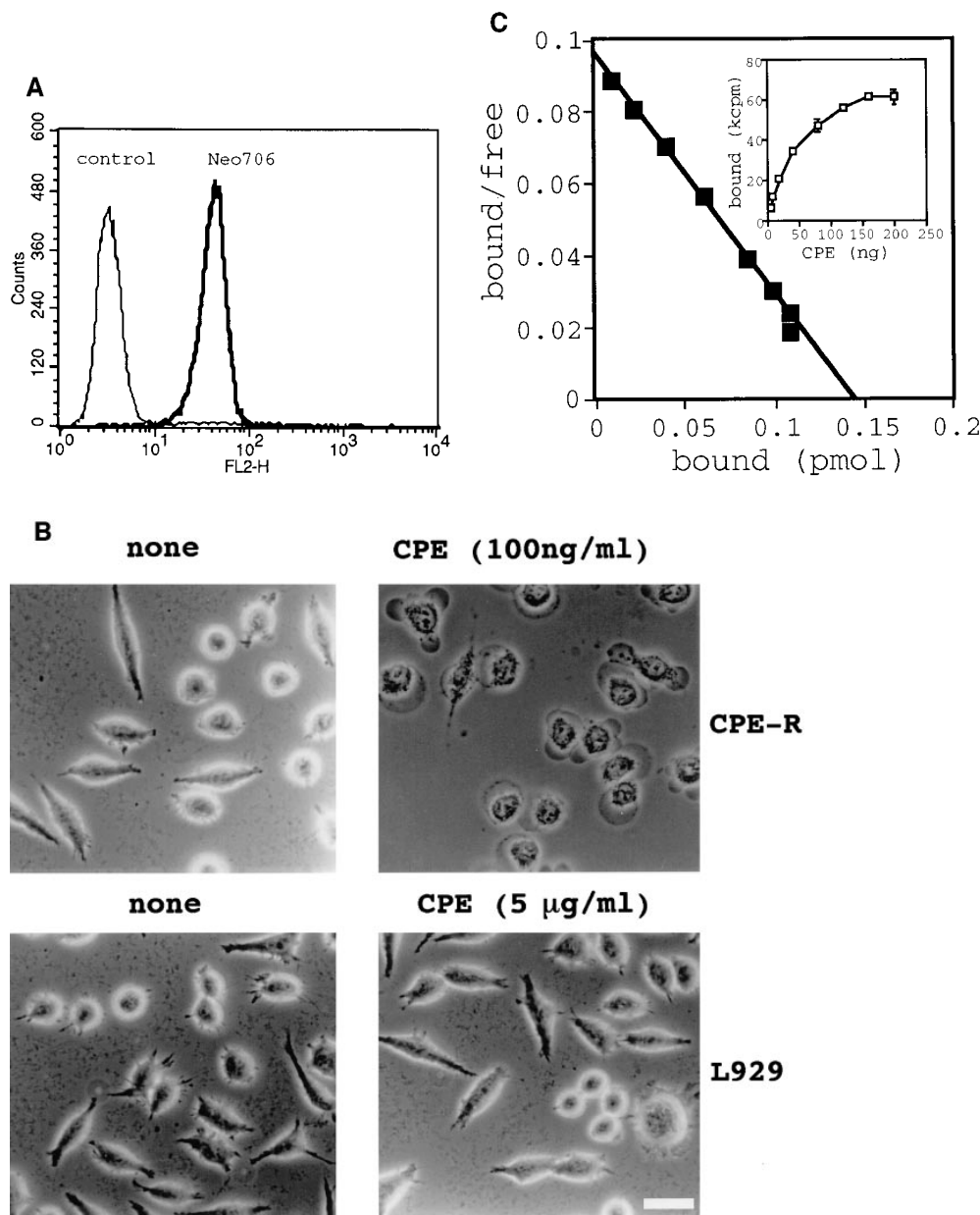
### Both CPE Binding Ability and CPE Sensitivity of an L929 Cell Line Stably Expressing CPE-R

To determine whether the CPE-R product was functional, we established an L929 cell line stably expressing CPE-R (designated as the Neo706 cell line). Flow cytometric analysis revealed that the H<sub>10</sub>PER probe specifically bound to the cell surface of Neo706 cells (Fig. 5 A, thick line). Then we examined the CPE sensitivity of the Neo706 cell line. Purified CPE at 100 ng/ml caused Neo706 cells to form bleb balloons (compare Fig. 5 B, upper left with right)





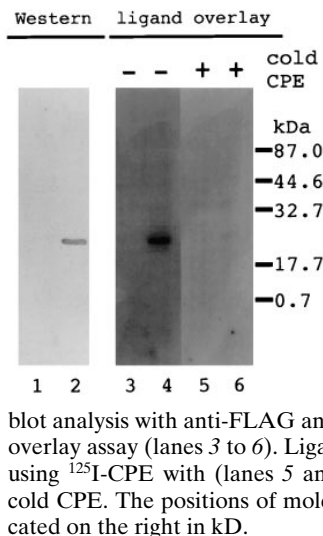
**Figure 4.** Northern blot analysis of *CPE-R*. Total RNA samples (15  $\mu$ g/lane) from Vero (lane 1), K562 (lane 2), Hep3B (lane 3), JY (lane 4), and Intestine 407 (lane 5) cell lines were subjected to Northern blot analysis. The blots were hybridized with  $^{32}$ P-labeled DNA probes from the entire sequence of clone 706 containing *CPE-R* cDNA (top). The blots were rehybridized with  $^{32}$ P-labeled human EF-1 $\alpha$  probe (bottom) to confirm the amounts and integrities of the samples. The positions of 28 and 18S ribosomal RNA are indicated on the right.



**Figure 5.** Functional analysis of stable cell lines expressing the CPE receptor. (A) Clonal L929 cell lines expressing the CPE receptor were isolated as described in Materials and Methods. Expression of the *CPE-R* gene product was examined by flow cytometric analysis in a typical cell line expressing *CPE-R* (thick line, indicated as *Neo706*) and parental L929 cell line (thin line, indicated as *control*), as shown in Fig. 2. Cell numbers are shown on the ordinate and relative fluorescence on a log scale on the abscissa. (B) Comparison of sensitivities of typical cell line expressing the CPE receptor (top) and the parental L929 cell line (bottom) to CPE. The cells were not treated with CPE (top and bottom, denoted as *none*) or with CPE at 100 ng/ml (top, denoted as *CPE*), or 5  $\mu$ g/ml (lower right, denoted as *CPE*) in DME supplemented with 10% FCS for 30 min at 37°C. Bar, 30  $\mu$ m. (C) Scatchard plot analysis of the binding of  $^{125}$ I-CPE to the L929 cell line expressing *CPE-R*. Scatchard plot analysis giving a  $K_d$  value of  $1.49 \times 10^8 \text{ M}^{-1}$  for  $^{125}$ I-CPE binding. (Inset) Various concentrations of  $^{125}$ I-CPE were incubated with the L929 cell line expressing *CPE-R* ( $0.4 \times 10^6$  cells each concentration), and specific binding was determined as described before (Horiguchi et al., 1985). Symbols represent means  $\pm$  SD ( $n = 6$ ).

### Inclusion of the CPE-R Gene Product in the CPE-induced Large Complex

After binding to the cell surface, CPE is reported to form a large hydrophobic complex, which may possibly constitute the pore in the cell membrane (Wieckowski et al., 1994). Therefore we examined whether CPE formed a complex containing the CPE receptor. For this the 706FLAG cells were labeled with [ $^{35}$ S]methionine, treated with CPE or H<sub>10</sub>PER at 4° or 37°C for 30 min, and then lysed. The cell lysates were treated with anti-FLAG antibody, and the precipitates were separated by SDS-PAGE. The CPE receptor migrated as a band of ~22 kD (Fig. 7, A and B, lanes 5–7). In addition to this band, a higher molecular weight protein band was observed when CPE was incubated with the 706FLAG cells (Fig. 7 A, lanes 5 and 6), suggesting that cellular proteins form a complex with CPE. This band was intense when the 706FLAG cells were treated with CPE at 37°C, but was not detectable when the



**Figure 6.** Specific and direct binding of CPE to the CPE-receptor. L929 cell lines expressing the CPE receptor with FLAG tag peptide at the COOH-terminal end were established as described in Materials and Methods, and a typical cell line (706FLAG) was selected. Cell lysates were prepared from the parental L929 (lanes 1, 3, and 5) and 706FLAG (lanes 2, 4, and 6) cell lines and subjected to Western

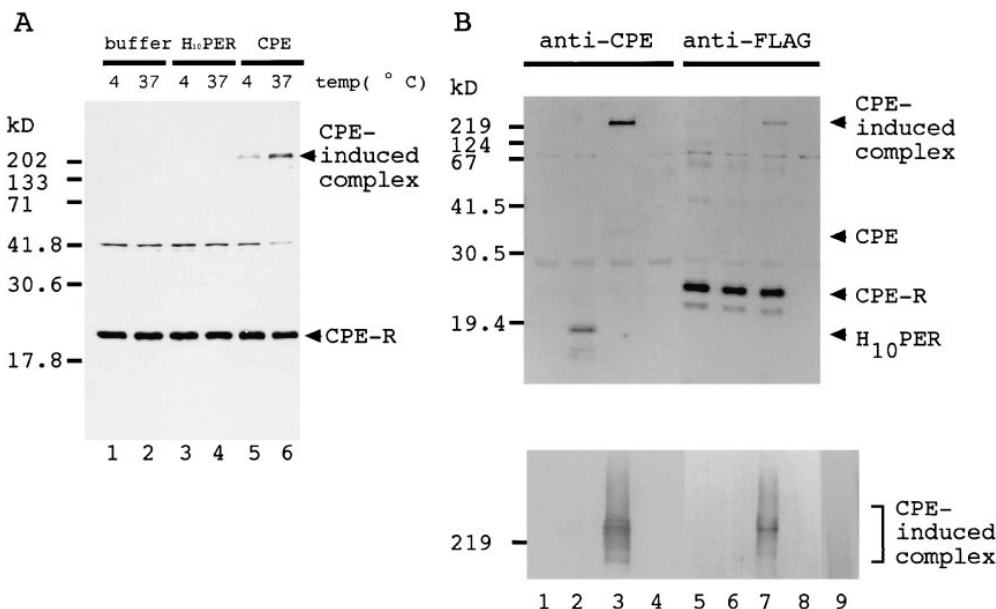
blot analysis with anti-FLAG antibody (lanes 1 and 2) and ligand overlay assay (lanes 3 to 6). Ligand overlay assay was carried out using  $^{125}\text{I}$ -CPE with (lanes 5 and 6) or without (lanes 3 and 4) cold CPE. The positions of molecular weight standards are indicated on the right in kDa.

706FLAG cells were treated with  $\text{H}_{10}\text{PER}$  (Fig. 7 A, lanes 3 and 4). An additional band of  $\sim 42$  kD was precipitated from all the samples. This was probably a protein that reacted nonspecifically with anti-FLAG resin, because it was also detected in a precipitate prepared from the parental L929 cell line (data not shown). Decrease in intensity of this band was probably due to leakage of cell contents on CPE-induced cell lysis during the incubation (Fig. 7 A, lane 6). The components of this complex were examined by Western blot analysis of the immunoprecipitates. Anti-

FLAG antibody recognized the CPE receptor (Fig. 7 B, lanes 5–7, CPE-R) as well as the large complex (Fig. 7 B, lane 7). The complex also reacted with anti-CPE antibody (Fig. 7 B, lane 3). These results indicate that the complex contained at least CPE and the CPE receptor. As expected, neither the CPE receptor nor the large complex was detected in CPE-insensitive L929 cells treated with CPE (Fig. 7 B, lanes 4 and 8).  $\text{H}_{10}\text{PER}$  was also coprecipitated with the CPE receptor (Fig. 7 B, lane 2,  $\text{H}_{10}\text{PER}$ ), but the large complex was not formed (Fig. 7 B, lanes 2 and 6, and A, lanes 3 and 4). When CPE alone was subjected to SDS-PAGE, the large protein band was not detected, although it aggregated and migrated as broad bands (Fig. 7 B, lower panel, lane 9) as reported previously (Enders and Duncan, 1976). From these results we concluded that the complex was formed by specific interaction between CPE and the CPE receptor. The  $\text{NH}_2$ -terminal half of CPE, which is known to be necessary for cytotoxic activity, was essential for formation of the complex.

## Discussion

The present study describes a novel CPE receptor gene product, the expression of which confers CPE-resistant L929 cells with both ability to bind CPE and CPE sensitivity. This 22-kD receptor was essential for the cytotoxic action of CPE, as demonstrated by induction of its stable expression in CPE-insensitive L929 cells and showed all the characteristics of the CPE receptor so far reported. The present results also suggest that CPE acts through a unique mechanism.



**Figure 7.** Presence of both the CPE and CPE receptor in a CPE-induced high molecular weight complex. (A) 706 FLAG cells were labeled with  $^{35}\text{S}$ methionine and treated with buffer alone (lanes 1 and 2) or buffer containing either 140 nmol of  $\text{H}_{10}\text{PER}$  (lanes 3 and 4) or CPE (lanes 5 and 6) for 30 min at  $4^\circ\text{C}$  (odd-numbered lanes) or  $37^\circ\text{C}$  (even-numbered lanes). The cells were solubilized with PBS-0.5% NP-40, and the lysates were immunoprecipitated with anti-FLAG antibody. Precipitated proteins were separated by SDS-15% PAGE, and protein bands were located with Bioimaging Analyzer. The positions of molec-

ular weight standards are indicated on the left in kD. (B) 706FLAG cells (lanes 1–3 and 5–7), and parental L929 cells (lanes 4 and 8) were treated with buffer alone (lanes 1 and 5) or buffer containing either 140 nmol of  $\text{H}_{10}\text{PER}$  (lanes 2 and 6) or CPE (lanes 3, 4, 7, and 8). The cells were solubilized with PBS-0.5% NP-40 and immunoprecipitated with anti-FLAG antibodies. The precipitates were separated by SDS-15% PAGE (top) or SDS-2–15% gradient PAGE (bottom; note that only the relevant area is shown), transferred to a PVDF membrane, and then treated with biotinylated anti-CPE (lanes 1–4) or anti-FLAG (lanes 5–8) antibodies followed by alkaline phosphatase-conjugated streptavidin. As a control, 7 nmol of purified CPE was applied to SDS-2–15% polyacrylamide gradient gel, and then subjected to Western blotting with biotinylated anti-CPE antibody (bottom, lane 9). The positions of molecular weight standards are indicated on the left in kD.

### Functionality of the CPE-R Gene Product

Several groups have reported that CPE uses a high affinity binding site on the target cell membrane as a specific receptor (McDonel and McClane, 1979; McDonel, 1980; Horiguchi et al., 1985). The present results are consistent with these previous reports, because the 22-kD CPE receptor could act as a high affinity binding site ( $K_a = 1.49 \times 10^8 \text{ M}^{-1}$ ) for CPE. In addition, we demonstrated the in vitro binding of CPE to the CPE receptor by ligand overlay assay. Thus CPE binds directly and specifically to the CPE receptor as the initial step in CPE-induced intoxication.

On analysis, the primary structure of this small 22-kD CPE receptor was found to contain 4 putative transmembrane segments. No possible glycosylation site was identified in its primary amino acid sequence of the receptor, indicating that it is not modified by carbohydrate. In fact, treatment with tunicamycin did not significantly alter its electrophoretic mobility on SDS-PAGE (Katahira, J., N. Inoue, Y. Horiguchi, M. Matsuda, and N. Sugimoto, unpublished results). These characteristics of our cloned *CPE-R* gene product are consistent with reports that the sensitivities and the binding abilities of CPE-sensitive cells were not affected by neuraminidase treatment or the additions of various sugars to the media for binding reactions (Tollehaug et al., 1982; Wnek and McClane, 1986).

Most importantly, we showed here that the CPE-insensitive L929 cell line became CPE sensitive on expression of *CPE-R*. Thus we conclude that the cloned gene product is the functional CPE receptor.

### Induction by CPE of a Large Protein Complex Containing Both CPE and the CPE-Receptor

Wieckowski et al. (1994) detected a large hydrophobic complex after treatment of CPE-sensitive cells with CPE. By coimmunoprecipitation assay, we demonstrated here that a large complex contains both CPE and the 22-kD CPE receptor. This complex was fairly stable, not being dissociated completely by SDS treatment. The COOH-terminal half of CPE, which can bind directly to the CPE receptor but has no cytotoxic activity, did not form a complex. Moreover, formation of the complex was promoted by incubation of the cells with CPE at 37°C, a temperature at which the cytotoxic action is much greater than at 4°C (Horiguchi et al., 1985; McClane and Wnek, 1990). Thus this complex must be essential for CPE intoxication and form a CPE-induced pore in the cell membrane. Purified intact CPE shows an anomalous electrophoretic migration pattern, but the COOH-terminal fragment does not (Fig. 7 B). Thus we consider that the NH<sub>2</sub>-terminal half of CPE may have a role in self assembly but that it is not sufficient alone for formation of a defined structure. It is not yet clear whether the CPE receptor is the sole requisite for CPE intoxication. The interaction of CPE with its receptor might be necessary not only for the recognition of target cells but also for the regulated assembly of the CPE-induced complex in the plasma membrane. Thus we propose that CPE acts through a novel mechanism differing from those of other pore-forming toxins (for review see Bhakdi et al., 1996). Analyses of the stoichiometric features of CPE and its receptor in the complex, and investigations on its fur-

ther interaction with other components are now in progress in our laboratory.

### Similarities of the CPE-R Gene Product to Several Other Gene Products with No Known Function

The *CPE-R* gene product shows sequence similarities to the rat androgen withdrawal apoptosis protein RVP1 (Briehl and Miesfeld, 1991) and mouse oligodendrocyte specific protein (these sequence data are available from GenBank/EMBL/DDBJ under accession number U19582). These have been reported to be expressed in a highly restricted manner: *RVPI* gene is expressed in ventral prostate cells after withdrawal of androgen from the culture medium in vitro or castration in vivo (Briehl and Miesfeld, 1991); oligodendrocyte specific protein is expressed specifically in oligodendrocyte. *CPE-R* was constitutively expressed in various cell lines tested in this study and in tissues including kidney, liver, and intestine (Katahira, J., N. Inoue, Y. Horiguchi, M. Matsuda, and N. Sugimoto, unpublished results). On searching the expressed sequence tag data base, we also obtained *CPE-R* and putative *RVPI* homologues of human and mouse origin. We found that the human CPE receptor homologue showed higher homology to monkey CPE receptor (99.0% identity, 99.5% similarity) than to human RVP1 (70.9% identity, 96.6% similarity) and that the mouse CPE receptor showed higher homology to the monkey CPE receptor (83.8% identity, 98.6% similarity) than to rat RVP1 (66.8% identity, 94.6% similarity; Katahira, J., N. Inoue, Y. Horiguchi, M. Matsuda, and N. Sugimoto, manuscript in preparation). Thus we consider that RVP1 may be a different gene product, although CPE receptor and RVP1 share a structural similarity and might be members of a functionally identical gene family. Further functional analysis of the *CPE-R* product may provide a clue to not only the physiological function of the CPE receptor but also the functions of RVP1 and the oligodendrocyte specific protein.

We are indebted to Dr. T. Kinoshita and members of the Department of Immunoregulation for their helpful discussions and providing plasmids. We are grateful to the following persons of Research Institute for Microbial Diseases, Osaka University; Dr. H. Nojima (Department of Molecular Genetics) for helpful suggestions on library construction and Drs. N. Wakamiya (Department of Viral Infections) and K. Nagayama (Department of Bacterial Infections) for providing cell lines. We are also grateful to Dr. T. Asao (Osaka Prefectural Institute of Public Health) for providing the *C. perfringens* strain. We also thank K. Nakamura, Central Laboratory, Research Institute for Microbial Diseases, Osaka University, for cell sorting. We are grateful to Dr. S. Kozaki (University of Osaka Prefecture) for critical reading of this manuscript.

Received for publication 12 November 1996 and in revised form 17 December 1996.

### References

- Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Bhakdi, S., B. Bayley, A. Valeva, I. Walev, B. Walker, U. Weller, M. Kehoe, and M. Palmer. 1996. Staphylococcal alpha-toxin, streptolysin-O, and *Escherichia coli* hemolysin: prototypes of pore-forming bacterial cytolysins. *Arch. Mikrobiol.* 165:73-79.
- Briehl, M.M., and R.L. Miesfeld. 1991. Isolation and characterization of transcripts induced by androgen withdrawal and apoptotic cell death in the rat ventral prostate. *Mol. Endocrinol.* 5:1381-1388.
- Czczulin, J.R., P.C. Hanna, and B.A. McClane. 1993. Cloning nucleotide sequencing and expression of the *Clostridium perfringens* enterotoxin gene in



- Escherichia coli*. *Infect. Immun.* 61:3429–3439.
- Enders, G.L., and C.L. Duncan. 1976. Anomalous aggregation of *Clostridium perfringens* enterotoxin under dissociation conditions. *Can. J. Microbiol.* 22: 1410–1414.
- Gubler, U., and B.J. Hoffmann. 1983. A simple and very efficient method for generating cDNA libraries. *Gene (Amst.)* 25:263–269.
- Hanna, P.C., T.A. Mietzner, G.K. Schoolnik, and B.A. McClane. 1991. Localization of the receptor-binding region of *Clostridium perfringens* enterotoxin utilizing cloned toxin fragments and synthetic peptides. The 30 C-terminal amino acids define a functional binding region. *J. Biol. Chem.* 266:11037–11043.
- Hanna, P.C., E.H. Wieckowski, T.A. Mietzner, and B.A. McClane. 1992. Mapping of functional regions of *Clostridium perfringens* type A enterotoxin. *Infect. Immun.* 60:2110–2114.
- Higgins, D.G., and P.M. Sharp. 1989. Fast and sensitive multiple sequence alignments on a microcomputer. *Comput. Appl. Biosci.* 5:151–153.
- Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* 26:365–369.
- Horiguchi, Y., T. Uemura, S. Kozaki, and G. Sakaguchi. 1985. The relationship between cytotoxic effect and binding to mammalian cultured cells of *Clostridium perfringens* enterotoxin. *FEMS Microbiol. Lett.* 28:131–135.
- Horiguchi, Y., T. Uemura, Y. Kamata, S. Kozaki, and G. Sakaguchi. 1986. Production and characterization of monoclonal antibodies to *Clostridium perfringens* enterotoxin. *Infect. Immun.* 52:31–35.
- Horiguchi, Y., T. Akai, and G. Sakaguchi. 1987. Isolation and function of a *Clostridium perfringens* enterotoxin fragment. *Infect. Immun.* 55:2912–2915.
- Ikeda, S., J. Oka, and T. Nagao. 1991. Effects of four diltiazem stereoisomers on binding of d-cis-[<sup>3</sup>H]diltiazem and (+)-[<sup>3</sup>H]PN200-110 to rabbit T-tubule calcium channels. *Eur. J. Pharmacol.* 208:199–205.
- Kozak, M. 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* 15:8125–8148.
- Kyte, J., and R.F. Doolittle. 1982. A simple method for displaying the hydrophobic character of a protein. *J. Mol. Biol.* 157:105–132.
- Manser, E., T. Leung, C. Monfries, M. Teo, C. Hall, and L. Lim. 1992. Diversity and versatility of GTPase activating proteins for the p21 rho subfamily of ras G proteins detected by novel overlay assay. *J. Biol. Chem.* 267:16025–16028.
- Manser, E., T. Leung, H. Salihuddin, Z. Zhao, and L. Lim. 1994. A brain serine/threonine protein kinase that inhibits the GTPase activity of p21<sup>cdc42</sup>. *Nature (Lond.)* 367:40–46.
- Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. Mol. Biol.* 3:208–218.
- Matsuda, M., and N. Sugimoto. 1979. Calcium-independent and dependent steps in action of *Clostridium perfringens* enterotoxin on HeLa and Vero cells. *Biochem. Biophys. Res. Commun.* 91:629–636.
- Matsuda, M., K. Ozutsumi, H. Iwahashi, and N. Sugimoto. 1986. Primary action of *Clostridium perfringens* type A enterotoxin on HeLa and Vero cells in the absence of extracellular calcium: rapid and characteristic changes in membrane permeability. *Biochem. Biophys. Res. Commun.* 141:704–710.
- McClane, B.A., and J.L. McDonel. 1979. The effects of *Clostridium perfringens* enterotoxin on morphology, viability, and macromolecular synthesis in Vero cells. *J. Cell. Physiol.* 99:191–199.
- McClane, B.A., and J.L. McDonel. 1981. Protective effects of osmotic stabilizers on morphological and permeability alterations induced in Vero cells by *Clostridium perfringens* enterotoxin. *Biochim. Biophys. Acta.* 641:401–409.
- McClane, B.A., and A.P. Wnek. 1990. Studies of *Clostridium perfringens* enterotoxin action at different temperatures demonstrate a correlation between complex formation and cytotoxicity. *Infect. Immun.* 58:3109–3115.
- McClane, B.A., A.P. Wnek, K.I. Hulkower, and P.C. Hanna. 1988a. Divalent cation involvement in the action of *Clostridium perfringens* type A enterotoxin. *J. Biol. Chem.* 263:2423–2435.
- McClane, B.A., P.C. Hanna, and A.P. Wnek. 1988b. *Clostridium perfringens* type A enterotoxin. *Microb. Pathog.* 4:317–323.
- McDonel, J.L. 1980. Binding of *Clostridium perfringens* 125I-enterotoxin to rabbit intestinal cells. *Biochemistry.* 19:4801–4807.
- McDonel, J.L., and C.L. Duncan. 1975. Histopathological effect of *Clostridium perfringens* enterotoxin in the rabbit ileum. *Infect. Immun.* 12:1214–1218.
- McDonel, J.L., and B.A. McClane. 1979. Binding versus biological activity of *Clostridium perfringens* enterotoxin in Vero cells. *Biochem. Biophys. Res. Commun.* 87:497–504.
- McDonel, J.L., L.W. Chang, J.G. Pounds, and C.L. Duncan. 1978. The effect of *Clostridium perfringens* enterotoxin in rat and rabbit ileum: an electron microscopic study. *Lab. Invest.* 39:210–217.
- Ohishi, K., Y. Kurimoto, N. Inoue, Y. Endo, J. Takeda, and T. Kinoshita. 1996. Cloning and characterization of the murine GPI anchor synthesis gene *Pigf*, a homologue of the human *PIGF* gene. *Genomics.* 34:340–346.
- Sakaguchi, G., T. Uemura, and H. Riemann. 1973. Simplified method for purification of *Clostridium perfringens* type A enterotoxin. *Appl. Microbiol.* 27: 762–767.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Senda, T., N. Sugimoto, Y. Horiguchi, and M. Matsuda. 1995. The enterotoxin of *Clostridium perfringens* type A binds to the presynaptic nerve endings in neuromuscular junctions of mouse phrenic nerve-diaphragm. *Toxicol.* 33: 499–506.
- Stark, R.L., and C.L. Duncan. 1971. Purification and biochemical properties of *Clostridium perfringens* type A enterotoxin. *Infect. Immun.* 4:89–96.
- Sugii, S., and Y. Horiguchi. 1988. Identification and isolation of the binding substance for *Clostridium perfringens* enterotoxin on Vero cell. *FEMS Microbiol. Lett.* 52:85–90.
- Sugimoto, N., K. Ozutsumi, and M. Matsuda. 1985. Morphological alterations and changes in cellular cations induced by *Clostridium perfringens* type A enterotoxin in tissue culture cells. *Eur. J. Epidemiol.* 1:264–273.
- Sugimoto, N., M. Takagi, K. Ozutsumi, S. Harada, and M. Matsuda. 1988. Enterotoxin of *Clostridium perfringens* type A forms ion-permeable channels in a lipid bilayer membrane. *Biochem. Biophys. Res. Commun.* 156:551–556.
- Takahashi, M., N. Inoue, K. Ohishi, Y. Maeda, N. Nakamura, Y. Endo, T. Fujita, J. Takeda, and T. Kinoshita. 1996. PIG-B, a membrane protein of the endoplasmic reticulum with a large luminal domain, is involved in transferring the third mannose of the GPI anchor. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:4254–4261.
- Tolleshaug, H., R. Skjelkvale, and T. Berg. 1982. Quantitation of binding and subcellular distribution of *Clostridium perfringens* enterotoxin in rat liver cells. *Infect. Immun.* 37:486–491.
- Uetsuki, T., A. Naito, S. Nagata, and Y. Kaziro. 1989. Isolation and characterization of the human chromosomal gene for polypeptide chain elongation factor-1 alpha. *J. Biol. Chem.* 264:5791–5798.
- Watanabe, R., T. Kinoshita, R. Masaki, A. Yamamoto, J. Takeda, and N. Inoue. 1996. PIG-A and PIG-H, which participate in glycosylphosphatidylinositol anchor biosynthesis, form a protein complex in the endoplasmic reticulum. *J. Biol. Chem.* 271:26868–26875.
- Wieckowski, E.U., A.P. Wnek, and B.A. McClane. 1994. Evidence that an ~50 kDa mammalian plasma membrane protein with receptor-like properties mediates the amphiphilicity of specifically-bound *Clostridium perfringens* enterotoxin. *J. Biol. Chem.* 269:10838–10848.
- Wnek, A.P., and B.A. McClane. 1983. Identification of a 50,000 Mr protein from rabbit brush border membranes that binds *Clostridium perfringens* enterotoxin. *Biochem. Biophys. Res. Commun.* 112:1099–1105.
- Wnek, A.P., and B.A. McClane. 1986. Comparison of receptors for *Clostridium perfringens* type A and cholera enterotoxins in rabbit intestinal brush border membranes. *Microb. Pathog.* 1:89–100.