The 27-kD Diphtheria Toxin Receptor-associated Protein (DRAP27) from Vero Cells Is the Monkey Homologue of Human CD9 Antigen: Expression of DRAP27 Elevates the Number of Diphtheria Toxin Receptors on Toxin-sensitive Cells

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Abstract. Diphtheria toxin (DT) receptor associates with a 27-kD membrane protein (DRAP27) in monkey Vero cells. A cDNA encoding DRAP27 was isolated, and its nucleotide sequence was determined. The deduced amino acid sequence revealed that DRAP27 is the monkey homologue of human CD9 antigen. DRAP27 is recognized by CD9 antibodies. A human-mouse hybrid cell line (3279-10) possessing human chromosome 5, sensitive to DT, but not expressing CD9 antigen, was used for transfection experiments with DRAP27. When the cloned cDNA encoding DRAP27 was transiently expressed in 3279-10 cells, the total DT binding capacity was three to four times higher than that of untransfected controls. Transfectants stably expressing DRAP27 have an increased number of DT binding sites on the cell surface. Furthermore, the transfectants are 3–25 times more sensitive to DT than untransfected cells, and the sensitivity of these cells to DT is correlated with the number of DRAP27 molecules on the surface. However, when the cloned cDNA was introduced into mouse cell lines that do not express DT receptors, neither an increased DT binding nor enhancement of DT sensitivity was observed. Hence, we conclude that DRAP27 itself does not bind DT, but serves to increase DT binding and consequently enhances DT sensitivity of cells that have DT receptors. 12 proteins related to DRAP27/CD9 antigen were found through homology search analysis. These proteins appear to belong to a new family of transmembrane proteins.

DIPHTHERIA toxin (DT),[1] secreted by *Corynebacterium diphtheriae*, is a cytotoxic protein (M_r = 58,342) that inhibits cellular protein synthesis in eukaryotes by inactivating elongation factor 2 through ADP ribosylation (for reviews, see references 18 and 48). Entry of the toxin, or at least the A fragment, into the cytoplasm is required for the cytotoxic action to occur (65). The toxin binds to a specific receptor on the cell surface (15, 37) and is then internalized by receptor-mediated endocytosis (42, 45). A conformational change of the toxin molecule takes place in an acidic compartment, resulting in the interaction of hydrophobic domains of the toxin molecule with the lipid bilayer of the compartment (10, 11, 14, 52). Finally, the enzymatically active A fragment is translocated to the cytosol (43, 44), where it inactivates elongation factor 2. The sensitivity of cells to DT varies among species. Cells from many mammals, including humans and monkeys, are sensitive to DT, but those from rats and mice are not (40). The difference in DT sensitivity among cells is primarily determined by the number of DT-specific receptors on the surface (31, 36, 41).

The Vero cell line, derived from monkey kidney, is one of the cell lines most sensitive to DT (40). The use of Vero cells has facilitated biochemical studies of the DT receptor. Chemical cross-linking of the protein(s) on the cell surface with DT followed by immunoprecipitation with anti-DT antibody indicated that a 10–20-kD membrane protein from Vero cells was a candidate for the DT receptor (15). Using a solubilized fraction of Vero cell membrane, we showed that a 14.5-kD membrane protein (DTR14.5) is the DT receptor, or at least the binding moiety (37). In subsequent studies, DTR14.5 was purified (38). Furthermore, it was shown that DTR14.5 forms a complex or associates with another protein. In gel filtration studies of the solubilized membrane fraction, DT receptor eluted in fractions corresponding to a larger than expected size (37, 38). Additional evidence came from studies using a mAb that inhibits the binding of DT to Vero cells (28). This mAb, called 007, does not recognize DTR14.5 itself, but rather a 27-kD membrane protein. Studies using mAb 007 showed that this 27-kD membrane

1. *Abbreviations used in this paper*: DRAP27, 27-kD diphtheria toxin receptor-associated protein; DT, diphtheria toxin; RDS, retinal degeneration slow proteins.

protein (DRAP27) associates with DTR14.5. In addition, a correlation was found between the sensitivity of a cell line to DT and the number of DRAP27 molecules on the cell surface.

We describe here the cloning of the cDNA encoding DRAP27. The nucleotide sequence data and other evidence show that DRAP27 is the monkey homologue of human CD9 (cluster of differentiation) antigen. Transfection experiments using the cloned cDNA indicate that DRAP27 indeed plays a role in the binding of DT to cells and the sensitivity to DT. Moreover, homology search analysis indicates that the DRAP27/CD9 antigen belongs to a new family of transmembrane proteins.

Materials and Methods

Enzymes and Chemicals

Restriction enzymes and other enzymes for DNA manipulation were purchased from Toyobo Co., Ltd. (Osaka, Japan). A cDNA synthesis kit was purchased from Amersham Corp. (Arlington Heights, IL). [α-^{32}P]dCTP and [^{125}I]NaI were from New England Nuclear (Boston, MA).

Antibodies

mAb 007 was produced and purified in our laboratory as described previously (28). Mouse CD9 mAbs ALB6, BU16, MAB1206, and TP82 were purchased from Nichirei Co., Ltd. (Tokyo, Japan), The Binding Site, Inc. (San Diego, CA), Chemical International Inc. (Temecula, CA), and MBL Co., Ltd. (Nagoya, Japan), respectively. Affinity-purified goat anti-mouse IgG, FITC-conjugated goat anti-mouse IgG, and HRP-conjugated goat anti-mouse IgG were from Zymed Laboratories, Inc. (South San Francisco, CA).

Preparation of the cDNA Library

Total RNA from monkey Vero cells was prepared by the SDS/phenol method and poly(A)$^+$ RNA was purified by oligo (dT)-cellulose chromatography (51). Double-stranded DNA was prepared from 5 μg poly(A)$^+$ RNA using a cDNA synthesis kit. The cDNAs were ligated to 8- (5′ CTGGAAAG) and 12-mer (5′ CTTTCCAGCACA) phosphorylated BstXI linkers (2). Excess linkers and cDNAs of <200 bp were removed using a Select-6L spin column from 5 Prime 3 Prime, Inc. (West Chester, PA). The size-fractionated cDNAs were then ligated into the CDM8 expression vector (56), which had been digested with BstXI and recovered from a low-melting-point agarose gel. Finally, the ligation mixtures were introduced into *Escherichia coli* MC1061/p3, resulting in 1×10^5 independent clones.

DNA Preparation

Pools of colonies were cultured on LB agar plates. Plasmid DNA was isolated by the alkaline lysis method and purified either by CsCl-ethidium bromide gradient centrifugation or by phenol/chloroform extraction and ethanol precipitation (51).

Screening of the Transfectant of COPS Cells

Polyoma virus-transformed murine COPS cells were used for screening of the cDNA library. Plasmid DNA was introduced into COPS cells by the DEAE-dextran method, with chloroquine treatment (6). 48 h after transfection, COPS cells were washed once with binding medium (MEM-NEAA supplemented with 10% bovine calf serum and 20 mM Hepes, pH 7.2), incubated with mAb 007 at 250 ng/ml in binding medium at 37°C for 1 h, washed twice with washing buffer (PBS supplemented with 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mg/ml BSA), and once with binding medium. Then the cells were further incubated at 37°C for 1 h with ^{125}I-labeled goat anti-mouse IgG at 500 ng/ml in binding medium containing 2% goat serum. Goat anti-mouse IgG was labeled with ^{125}I using Enzymobeads (Bio-Rad Laboratories, Richmond, CA) as described previously (28). The labeled antibody had a specific activity of 1.1-1.3 $\times$ 10^4 cpm/ng protein. Binding of the radiolabeled antibody was measured either by autoradiography or by direct counting with a γ-counter. For autoradiography, transfectants were washed twice with washing buffer and once with PBS supplemented with 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$, fixed with PBS containing 2% formaldehyde at ambient temperature for 1 h, and dried. The edge of the dish was cut off, and the flat portion was attached to x-ray film. The film was exposed with an intensifying screen for several days at −20°C and then developed. For counting with a γ-counter, transfectants were washed twice with washing buffer and once with binding medium, they were lysed with 0.1 N NaOH, and the radioactivity of the lysate was counted.

Western Blotting

Cells transfected with plasmid were detached from the plates with PBS containing 0.02% azide and 1 mM EDTA and then pelleted by centrifugation. The pellets were suspended in SDS gel sample buffer without reducing agent and boiled for 2 min. The samples were run on 12.5% SDS-PAGE and then transferred to a nitrocellulose filter (S&S BAS 85) by electrotransfer. The filter was blocked with TBS (20 mM Tris-HCl, pH 7.5, 0.1 M NaCl) containing 3% nonfat milk at 37°C for 1 h and then incubated with 5 μg/ml mAb 007 in TBS buffer and 1% nonfat milk at 4°C for 14 h. The filter was washed three times with TTBS (20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 0.05% Tween 20) at ambient temperature for 15 min and then incubated with HRP-conjugated goat anti-mouse IgG (diluted 1:2,000) in TBS with 1% nonfat milk at 37°C for 2 h, followed by three washes with TTBS. Finally, the filter was developed in 0.05% DAB in 50 mM Tris-HCl, pH 7.4, containing 0.03% H_2O_2.

Immunofluorescence Microscopy

Cells transfected with plasmid were detached from dishes and replated on cover slips. The cells on the cover slips were incubated with 100 μg/ml mAb 007 in binding medium at 4°C for 3 h. The cells were washed with washing buffer, incubated at 37°C for 2 h in FITC-conjugated goat anti-mouse antibody (diluted 1:2,000) in binding medium, and washed three times with washing buffer. The cover slips were mounted on a glass slide and observed under a fluorescence microscope.

DNA Sequencing

Both strands of the single clone pCT1843 were sequenced by the dideoxy-mediated chain termination method (54) after successive deletion by exonuclease Bal31 and subcloning into pUC18.

Stable Expression of DRAP27 in Human-Mouse Hybrid 3279-10 Cells

Human-mouse hybrid 3279-10 cells were grown in DME supplemented with 10% FBS and 300 μg/ml G418. To isolate stable transfectants expressing DRAP27, $\sim 10^6$ 3279-10 cells were cotransfected with 11 μg of pCT1843 and 3 μg of HSV-TK gene (pHSV106) using DEAE-dextran as described above. After 48 h, the medium was replaced with HAT medium supplemented with 300 μg/ml G418 (selection medium). Cells were then cultured for 7 d. Colonies growing in selection medium were picked and subjected to immunofluorescence staining to test the expression of DRAP27 on the surface of the cells. Colonies were subcloned until almost all cells from each colony were positive for DRAP27.

Assay for DT Cytotoxicity

The sensitivity of cells to DT was assayed by measuring the inhibition of protein synthesis by DT as described previously (36). In this study, cells (1×10^5) were seeded in 24-well trays and incubated for 16 h. Then cells were incubated with DT for 4 h at 37°C, followed by incubation in the presence of [^{3}H]leucine (2 μCi/ml) for 60 min. The radioactivity incorporated into TCA-insoluble material was measured.

Assay for the Binding of CRM197

CRM197 was labeled with Na^{125}I using Enzymobeads. The specific activity of the CRM197 used for this assay was 1.5-2.5 $\times$ 10^4 cpm/ng. Cells were seeded in multiwell trays and incubated for 16 h in a CO_2 chamber. Then the medium was replaced with binding medium. ^{125}I-CRM197 was added and the cells were incubated at 4°C for 6 h or for the times indicated. The cells were washed, lysed, and the cell-associated radioactivity was counted as described previously (35). Nonspecific binding of ^{125}I-CRM197

was assessed in the presence of a 1,000-fold excess of unlabeled DT. Specific binding was determined by subtracting the nonspecific binding from the total binding obtained with ^{125}I-CRM197 alone.

Results

Isolation of cDNA for DRAP27

Binding studies using ^{125}I-labeled anti-DRAP27 antibody 007 showed that monkey Vero cells express DRAP27 abundantly (28). To isolate the cDNA encoding DRAP27, we synthesized double-stranded cDNAs from mRNA of Vero cells and introduced them into the expression vector CDM8 (56). The cDNA library was divided into 10 pools of ~10,000 independent clones. The cDNAs in each pool were introduced into murine COPS cells and the cells were cultured for 48 h. The cells were incubated with mAb 007 followed by incubation with ^{125}I-labeled anti-mouse IgG, then subjected to autoradiography. One pool of the cDNA library that showed a positive signal was further divided into smaller groups, and these were introduced into COPS cells. Groups were screened by direct counting using a γ-counter. This procedure was repeated until a single clone was obtained, and the recombinant plasmid from this clone was named pCT1843.

To confirm that the cloned cDNA in pCT1843 encodes DRAP27, cell lysate of COPS cells transfected with pCT1843 was analyzed by Western blotting. Transfectants harboring pCT1843 showed a distinct band at 27 kD, which was identical to DRAP27 from Vero cell membrane. Neither mock transfectants nor cells transfected with CDM8 showed any band at all (data not shown). In addition, immunofluorescence staining showed that the 27-kD protein was localized on the surface of COPS cells transfected with pCT1843. These data indicate that pCT1843 encodes the complete structural gene for DRAP27.

DRAP27 Is the Monkey Homologue of Human CD9 Antigen

The nucleotide sequence of the 1,120-bp cDNA insert in pCT1843 was determined (data not shown; sequence data are available from EMBL/GenBank/DDBJ under accession number D10726). Only one open reading frame encoding a 228-amino acid protein (M_r = 25,868) was found. The nucleotide sequence from positions −3 to +4 is similar to the consensus sequence for initiation of translation (32). Homology search analysis revealed that the deduced amino acid sequence of DRAP27 differs at only two residues from human CD9 antigen (34). Asp-151 and Ile-178 in DRAP27 are replaced by Asn-151 and Val-178, respectively, in CD9 antigen (see Fig. 7).

A number of mAbs to CD9 antigen have been isolated (13, 58). We examined whether CD9 antibodies react with DRAP27. Cell lysates of COPS cells transfected with pCT1843 were subjected to Western blotting using a mouse anti-CD9 mAb, ALB6, as probe. The antibody showed a distinct band identical to DRAP27 (data not shown). We then tested whether anti-CD9 mAbs inhibit the binding of DT to Vero cells, as does the anti-DRAP27 antibody 007. Surprisingly, all four mouse mAbs to CD9 (ALB6, BU16, MAB1206, and TP82) inhibited the binding of CRM197 to Vero cells. Fig. 1 shows the inhibitory effect of ALB6 and TP82. Although the epitopes recognized by these antibodies are not characterized, the antibodies used were isolated independently. From these results, we conclude that DRAP27 is the monkey homologue of human CD9 antigen.

Inhibition of DT Toxicity by Antibody 007 Is Mediated by CD9 Antigen

We previously showed that the mAb 007 binds to various human cell lines (28). As shown in Fig. 2, antibody 007 inhibits the toxicity of DT to human HEL and FL cells. These results indicate that antibody 007 reacts with human CD9 antigen and suggest that human CD9 antigen also participates in DT intoxication.

The gene encoding CD9 antigen is located on human chromosome 12 (9), whereas the gene responsible for DT sensitivity, probably the gene for the DT receptor, is located on human chromosome 5 (20). The 3279-10 cell line, a human–mouse hybrid, contains only human chromosomes 5 and 22 (24), and thus should not express CD9 antigen. We confirmed by immunofluorescence staining that this cell line does not express CD9 antigen. As shown in Fig. 2, antibody 007 does not inhibit the toxicity of DT for 3279-10 cells. This result is consistent with the hypothesis that the inhibitory effect of antibody 007 is mediated by the binding of the antibody to DRAP27/CD9 antigen, which these cells lack.

Transfection of cDNA Encoding DRAP27 Enhances the Binding of DT to CD9 Antigen-negative Hybrid Cells

To study the role of DRAP27/CD9 antigen in DT binding

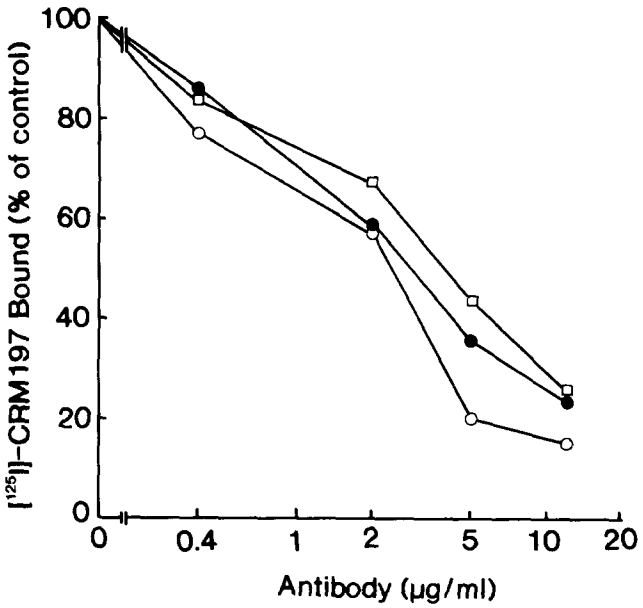

Figure 1. Inhibition of ^{125}I-labeled CRM197 binding to Vero cells by anti-CD9 monoclonal antibodies. Vero cells were incubated with various amounts of each antibody at 4°C for 2 h. ^{125}I-Labeled CRM197 (55 ng/ml) was added, and the cells were further incubated at 4°C for 4 h. The cells were washed, and the cell-associated radioactivity was counted. The results are expressed as percent of specific binding in the absence of antibody. (□) anti-CD9 monoclonal antibody ALB6; (○) TP82; (●) anti-DRAP27 antibody 007.

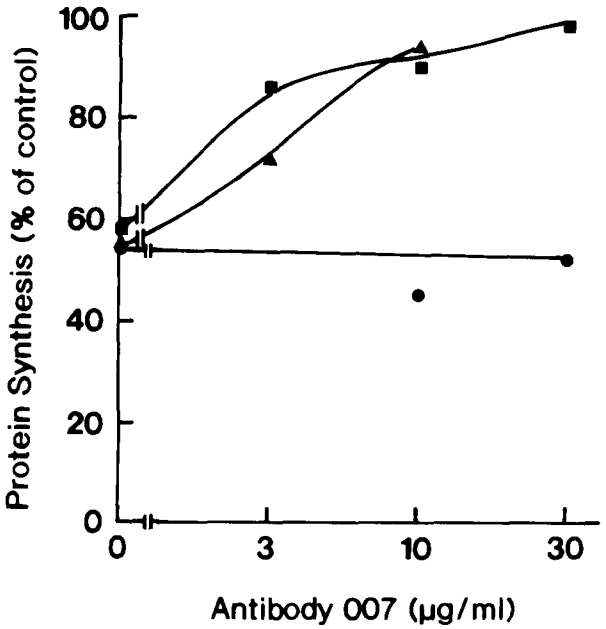

Figure 2. Effect of anti-DRAP27 monoclonal antibody 007 on the toxicity of DT for human FL cells, HEL cells, and human–mouse hybrid 3279-10 cells. Various concentrations of antibody 007 were added to cultures of FL cells (■), HEL cells (▲), and 3279-10 cells (●), and the cells were incubated at 37°C for 30 min. DT was added (5 ng/ml for HEL cells, 10 ng/ml for FL cells, and 3279-10 cells). The cells were further incubated for 2 h (FL cells and HEL cells) or 4 h (3279-10 cells), followed by incubation with 2 μCi/ml of [^{3}H]leucine for 60 min. The radioactivity incorporated into protein was measured. The data are expressed as percent of incorporation in the absence of toxin.

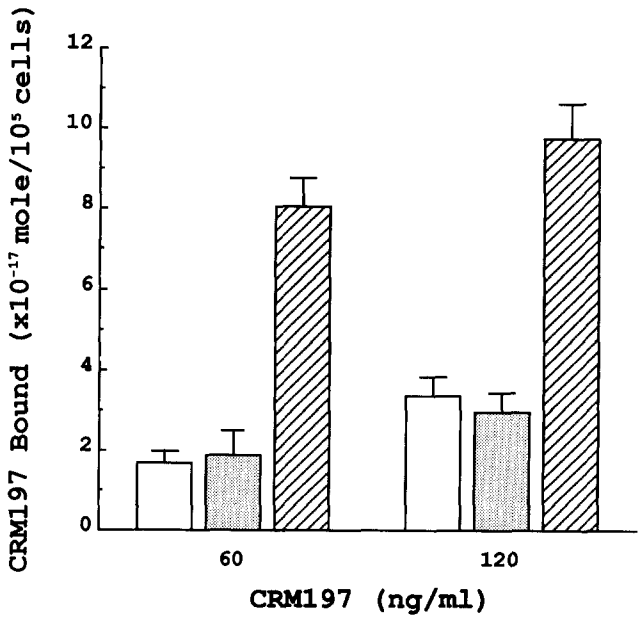

Figure 3. Increased binding of ^{125}I-labeled CRM197 to human–mouse hybrid 3279-10 cells transfected with the cDNA encoding DRAP27. 48 h after transfection, cells were incubated at 4°C for 6 h with the indicated concentrations of ^{125}I-labeled CRM197. Cells were then washed, and the radioactivity associated with the cells was counted. The data are expressed as specific binding. The values for nonspecific binding of ^{125}I-labeled CRM197 at 60 and 120 ng/ml were $\sim 2 \times 10^{-17}$ mol/10^5 cells and $\sim 4 \times 10^{-17}$ mol/10^5 cells, respectively. The mean values of duplicate samples are plotted in this figure. Bars indicate the deviation of the individual values from the mean: *open bars*, mock transfection; *dotted bars*, CDM8 vector; *hatched bars*, pCT1843.

more directly, pCT1843 was introduced into cells lacking DRAP27/CD9 antigen, and the binding activity was measured. The binding studies were carried out using CRM197, a mutant form of DT, because of its advantages for binding studies, as described previously (35, 37, 38).

COPS cells and L cells derived from mice have no DT receptors, thus no specific binding of ^{125}I-CRM197 is observed. We introduced pCT1843 into L cells or COPS cells. Binding of ^{125}I-CRM197 to the cells was tested 48 h after introduction of the plasmid. Although immunofluorescence staining showed strong expression of DRAP27 on 20–30% of the cells, no specific binding of ^{125}I-CRM197 was observed in either cell line (data not shown). This result is consistent with our observation that the DRAP27 molecule itself is not the DT-binding molecule (28).

Next, transfection experiments were performed using the human–mouse hybrid cell line, 3279-10. Because monkey and human cells express large amounts of endogenous DRAP27/CD9 antigen (28), it may be difficult to examine the effect of the expression of the cDNA encoding DRAP27 in such cells. The hybrid cell 3279-10 has DT receptors but does not express CD9 antigen. Thus, we used the 3279-10 cells as recipients in transfection experiments. As shown in Fig. 3, transfection of 3279-10 cells with pCT1843 increased specific binding of CRM197 three- to fourfold compared with untransfected control cells. Neither mock transfection nor transfection with the vector CDM8 had any effect. The transfection efficiency in this experiment was 20–30%, judged by expression of DRAP27 on the cell surface by immunofluorescence staining. This suggests that, in the cells expressing DRAP27, the specific binding of CRM197 may have been enhanced by about 10-fold.

Increased Sensitivity to DT in 3279-10 Cells Stably Expressing DRAP27

To assess the role of DRAP27/CD9 antigen more precisely, stable transfectants of 3279-10 cells expressing DRAP27 were isolated. Because 3279-10 cells already possess a neomycin resistance marker but lack the gene for thymidine kinase (24), 3279-10 cells were cotransfected with pCT1843 and pHSV106 plasmid. Colonies grown in HAT selection medium were tested for surface expression of DRAP27 by immunofluorescence staining, and several clones were isolated.

The sensitivity of each clone to DT was tested. The results are shown in Fig. 4. The parental 3279-10 cells were sensitive to DT, but their sensitivity is ~60 times less than that of monkey Vero cells when ED_{50} values are compared. Although the sensitivity differed from clone to clone, all the clones expressing DRAP27 showed increased sensitivity to DT: clones C15-2, C-1, and C7-3 were 22, 6, and 3 times more sensitive than 3279-10 cells, respectively. A stable clone of 3279-10 cells transfected with pHSV106 alone showed no increase in DT sensitivity (data not shown). Fur-

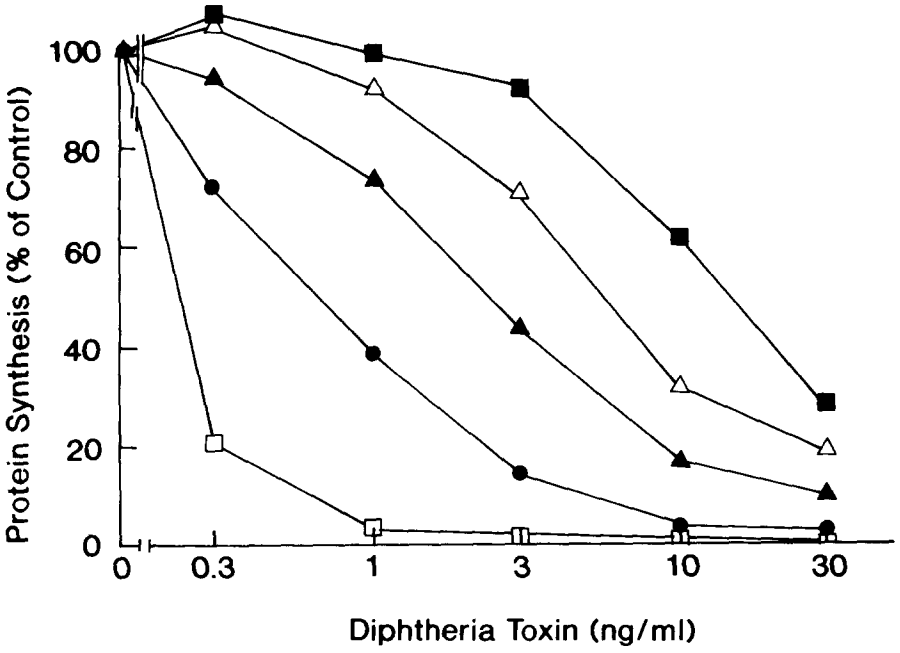

Figure 4. Inhibition of protein synthesis by DT in stable transfectants of 3279-10 cells expressing DRAP27, parental 3279-10 cells, and monkey Vero cells. Cells were incubated for 4 h at 37°C with various concentrations of DT, followed by incubation with [^{3}H]leucine for 1 h. The radioactivity incorporated in proteins were measured as described in Materials and Methods. Data are expressed as percent of protein synthesis for control without toxin. (●) clone C15-2 cells; (▲) clone C-1 cells; (△) clone C7-3 cells; (■) parental 3279-10 cells; (□) Vero cells.

thermore, the increased sensitivity to DT of clone C-1 was diminished by the addition of antibody 007, whereas the antibody did not affect the DT sensitivity of the parental 3279-10 cells (Fig. 5).

The number of DRAP27 molecules on the surface of each transfectant clone was determined by measuring the binding of ^{125}I-labeled antibody 007 to the cells. DT sensitivity, number of DT binding sites, and their Ka values are shown in Table I. The sensitivity of cells to DT, the number of DT binding sites, and the number of DRAP27 molecules on the surface of cells are correlated.

Transfection of mouse L cells and COPS cells with the cDNA of DRAP27 had no effect on the DT binding of these cells. We also examined the effect of expression of DRAP27 on the sensitivity of L cells to DT. To do this, we isolated stable transfectants of L cells expressing DRAP27 by the same procedures used with 3279-10 cells. L cells are ~4,400 times less sensitive to DT than 3279-10 cells. Although clone LC-14 expressed DRAP27 in amounts comparable to the transfectants of 3279-10 cells, the sensitivity to DT of these cells was similar to that of the parental L cells (Table I), indicating that the expression of DRAP27 in the absence of DT receptor does not enhance the sensitivity of cells.

DRAP27 Increases the Number of DT Receptors on the Surface of 3279-10 Cells

In transient expression experiments, DRAP27 enhanced DT

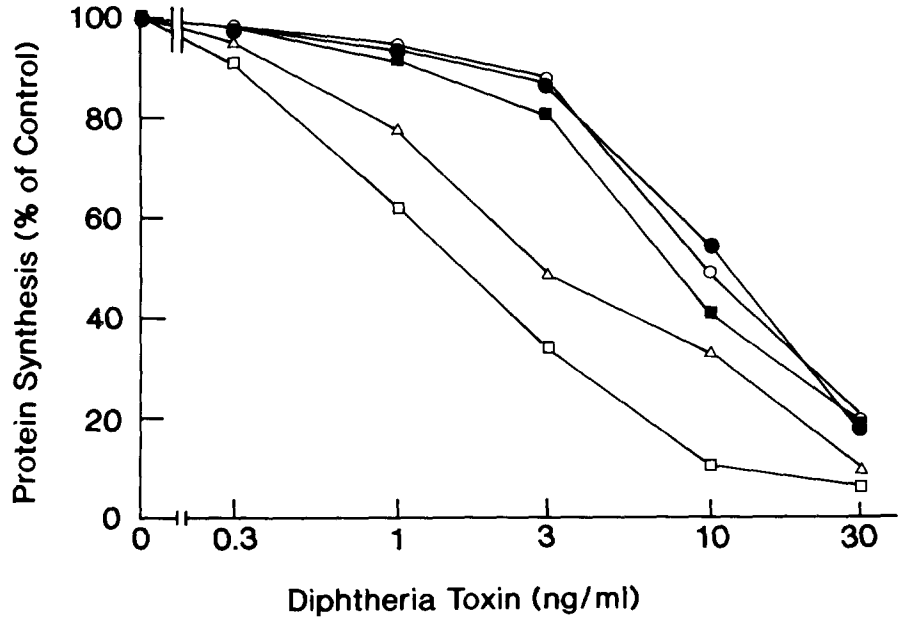

Figure 5. Effect of antibody 007 on the toxicity of DT for clone C-1 and 3279-10 cells. Cells were incubated at 37°C for 4 h with DT in the presence or absence of antibody 007, and the inhibition of protein synthesis by DT was measured. The data are expressed as percent of protein synthesis in the control (without DT). C-1 cells, (□) no antibody, (△) 10 μg/ml antibody, and (■) 30 μg/ml antibody 007; 3279-10 cells, (○) no antibody 007, (●) 30 μg/ml antibody 007.

Table I. Comparison of DRAP27 Expression, DT Sensitivity, and CRM197 Binding

Cells	DRAP27 expression*	DT sensitivity‡	CRM197 binding	Ka
	molecules/cell	*ng/ml*	*sites/cell*	
3279-10	0	14.5	500	
C7-3	2.5×10^6	5.6	1,300	
C-1	4.7×10^6	2.3	1,900	0.6×10^9
C15-2	1.2×10^7	0.65	2,600	1.3×10^9
Vero	5.6×10^6	0.23	19,500	1.0×10^9
L	0	6.4×10^4	ND	ND
LC-14	4.7×10^6	5.5×10^4	ND	ND

* Numbers of DRAP27 molecules on the cell surface were determined by binding experiments with a saturating amount (100 μg/ml) of ^{125}I-labeled antibody 007 at 4°C for 6 h.
‡ DT sensitivity was determined from the data of Fig. 4. The values are expressed as the concentration of toxin required to reduce the rate of protein synthesis to 50% of the control value (ED_{50}).

binding of 3279-10 cells. To examine the effect of DRAP27 on DT binding more closely, binding of ^{125}I-labeled CRM197 to stable transfectants was measured. As shown in Fig. 6, specific binding is enhanced in the transfectants over the entire concentration range tested. Specific binding of ^{125}I-labeled CRM197 to clones C15-2 and C-1 was ~5 and ~2.5 times higher, respectively, than that in 3279-10 cells. Nonspecific binding was not affected by the expression of DRAP27. Scatchard plot analysis indicated that C15-2 cells have 2,600 sites/cell with a Ka of 1.3×10^9 M^{-1}, while C-1 cells have 1,900 sites/cell with a Ka of 0.6×10^9 M^{-2} (Fig. 6 *B* and Table I). The values for Vero cells, which were determined at the same time, were 19,500 sites/cell and 1.0×10^9 M^{-1} (Table I). In the case of parental 3279-10 cells, nonspecific binding accounted for ~50% of the total binding; the data was not suited for Scatchard plot analysis, but we estimated the binding site number ~500 sites/cell.

Antigen Belongs to a New Family of Transmembrane Proteins

Proteins whose amino acid sequences showed significant homology (a SD >6 using the method of Pearson and Lipman [49]) to DRAP27 were screened from data banks. Eight proteins besides CD9 antigen showed significant homology to DRAP27. Proteins showing homology to DRAP27, including CD9 antigen, are shown in Table II. These proteins include TAPA-1, a target of antiproliferation antibody (47); CO-029, a human tumor-associated antigen (59); ME491/CD63 antigen, a human melanoma-associated antigen (4, 5, 27, 39); Sm23 and Sj23 (23-kD antigens of *Schistosoma mansoni* and *Schistosoma japonica*, respectively [21, 67]); CD37 and CD53 antigens, human leukocyte surface antigens (1, 16, 17, 55); and OX-44, a rat leukocyte antigen (8). The same screening mentioned above was performed again using the amino acid sequences of each of the nine proteins as a probe. Four additional sequences showed significant homology only to ME491/CD63 antigen. These proteins were normal human, bovine, mouse, and rat gene products corresponding to retinal degeneration slow (RDS) mutants (7, 19, 62, 63).

Alignment of the amino acid sequences of the proteins listed in Table II using the maximum parsimony method of Hein (25) is shown in Fig. 7. Because the RDS proteins did not show significant homology to any of the proteins other than ME491/CD63 antigen, RDS proteins are not listed in Fig. 7. The following structural similarities are found in all the proteins in Fig. 7, suggesting that they have the same topology in the membrane: (*a*) they contain three putative transmembrane domains in the NH_2-terminal portion, a long hydrophilic (or amphiphasic) domain, and one transmembrane domain in the COOH terminus, as judged by the hydropathy profiles calculated by the method of Kyte and Doolittle (33); (*b*) the proteins have no signal sequences; and (*c*) the possible N-glycosylation sites in all the proteins are located in the region between the first and second transmembrane domains and/or between the third and fourth transmembrane domains, except in TAPA-1. Furthermore, the Cys-Cys-Gly sequence at positions 152 to 154 in DRAP27 is conserved in all the proteins. These structural features and the Cys-Cys-Gly sequences are also found in all four RDS proteins. In the alignment of Fig. 7, identical as well as chemically similar amino acids conserved in all proteins are distributed in all four putative transmembrane domains, with

Table II. Proteins with Significant Homology to DRAP27

	Length in amino acids	Local homology
		%
DRAP27	228	100.0
CD9	228	99.1
TAPA-1	236	44.2
CO-029	237	31.7
CD37	281	29.3
CD53	219	26.2
OX-44	219	30.7
ME491/CD63	238	24.5
SM23	218	25.2
SJ23	218	23.3
ME491/CD63	238	100.0
h-RDS	346	19.0
b-RDS	346	23.2
r-RDS	346	22.1
m-RDS	346	23.8

Proteins whose amino acid sequence showed signifiant homology to that of DRAP27 (standard deviation >6) were screened from data banks (GenBank release 68, EMBL release 27, and SWISS PROT release 17) using the method of Pearson and Lipman (49). The RDS proteins showed significant homology to ME491/CD63 antigen but not to DRAP27 under these criteria. The local homology between ME491/CD63 and the RDS proteins are also shown.

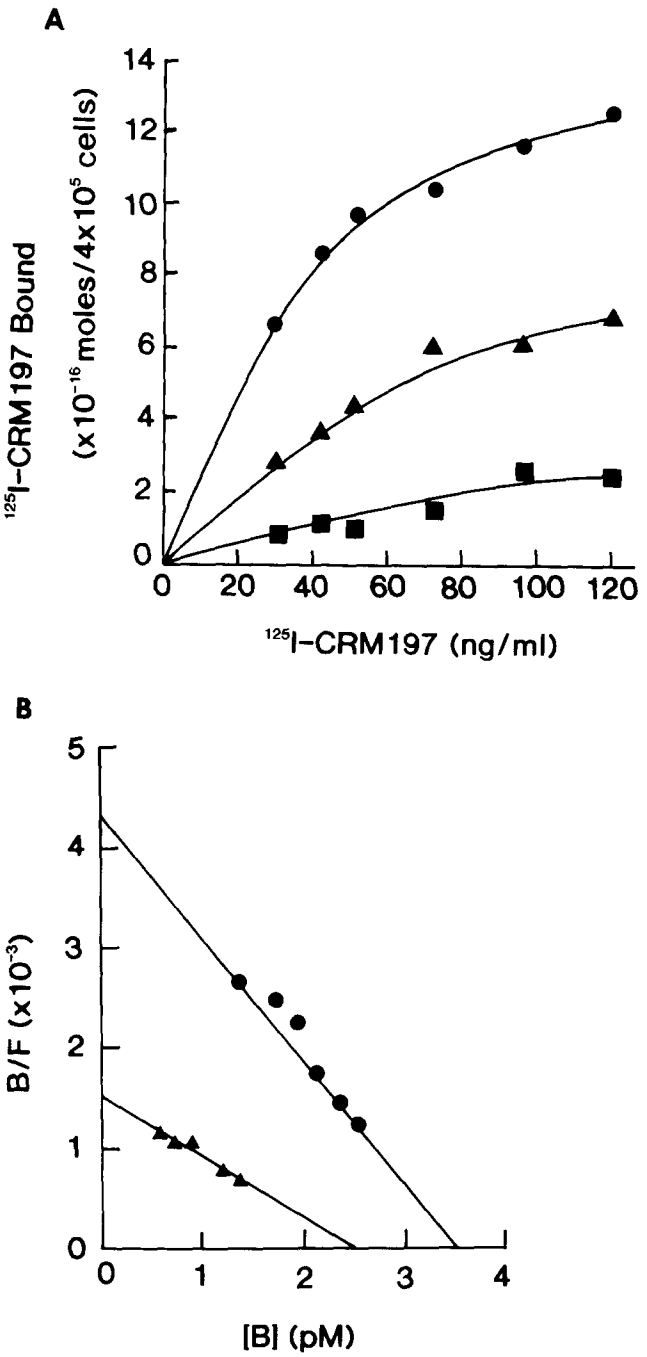

Figure 6. Binding of ^{125}I-labeled CRM197 to clone C15-2 cells, clone C-1 cells, and parental 3279-10 cells. (*A*) Cells (4 × 10^5/sample) were incubated at 4°C for 8 h with binding medium (0.5 ml) containing various concentrations of ^{125}I-labeled CRM197. Cells were then washed, and the radioactivity associated with the cells was determined. The data are expressed as specific binding. Nonspecific binding of ^{125}I-labeled CRM197 to C15-2 cells, C-1 cells, and 3279-10 cells was 10–16%, 20–26%, and 44–54% of the total binding, respectively. (*B*) Scatchard plot of the specific binding of ^{125}I-labeled CRM197 to C15-2 cells and C-1 cells. The lines were fitted by regression analysis. (●) clone C15-2 cells; (▲) clone C-1 cells; (■) 3279-10 cells.

the greatest similarity in the three NH_2-terminal putative transmembrane domains. In contrast, the long putative extracellular domains between the third and fourth transmembrane domains are highly divergent except for the Cys-Cys-Gly sequences. As the alignment of Fig. 7 was made by the maximum parsimony method of Hein (25), the phylogeny of these proteins was obtained simultaneously; that is shown in Fig. 8.

Discussion

In earlier studies, we showed that a mAb directed to a 27-kD membrane protein (DRAP27) inhibits the binding of DT to Vero cells and that DRAP27 associates with the DT receptor (DTR14.5) (28). We have cloned and sequenced the cDNA encoding DRAP27 and found that this protein is the monkey homologue of CD9 antigen, differing at only two amino acids. Moreover, four independently isolated mouse mAbs to CD9 antigen react with Vero cells and inhibit the binding of DT, and the CD9 antibody recognizes a 27-kD protein identical to DRAP27 when Western blotting analysis is performed using Vero cell membranes.

The anti-DRAP27 mAb, 007, inhibits the toxicity of DT to human cell lines, but it does not inhibit the cytotoxicity of DT to the human–mouse hybrid cell 3279-10 in which CD9 antigen is not expressed. These results confirm that the inhibitory effect of antibody 007 is due to the binding of the antibody to DRAP27/CD9 antigen. Although the direct evidence has not been obtained, we postulate that CD9 antigen associates with DT receptor molecules in human cell lines just as DRAP27 does in monkey Vero cells.

We previously suggested that DRAP27 may play a role in the binding and/or in later steps of DT entry into cells because of the correlation between the sensitivity of cell lines to DT and the number of DRAP27 molecules on the surface of the cells (28). We show here direct evidence by transfection of the cDNA encoding DRAP27 into human-mouse hybrid cell line lacking the gene for CD9 antigen. Expression of DRAP27 results in increased binding of DT and enhanced DT sensitivity. Thus, it is clear that DRAP27 has a positive effect on DT binding and intoxication. In contrast, transfection of the cDNA for DRAP27 into mouse cell lines did not increase DT binding or sensitivity. Therefore, it is also clear that the DRAP27/CD9 antigen itself is not a DT receptor.

Previous studies using human–mouse hybrid cells showed that a gene responsible for DT sensitivity is located on human chromosome 5 (20). The monochromosomal hybrid containing only human chromosome 5 is sensitive to DT. Significant binding of ^{125}I-labeled DT is observed, but the DT sensitivity and the amount of DT bound are lower than with human cells (3). These results are consistent with our present results. The gene responsible for DT sensitivity on chromosome 5 is likely to encode the DT receptor. The gene for CD9 antigen located on human chromosome 12 is probably also required for full expression of DT sensitivity in the mouse–human hybrid. Recently, DT-sensitive cells were isolated from toxin-resistant L_{tk}^- cells transfected with genomic DNA from Vero cells. Interestingly, the sensitivity of these cells was lower than that of Vero cells (46). This lower sensitivity to DT might be due to the absence of DRAP27.

How does DRAP27/CD9 enhance the binding of DT to

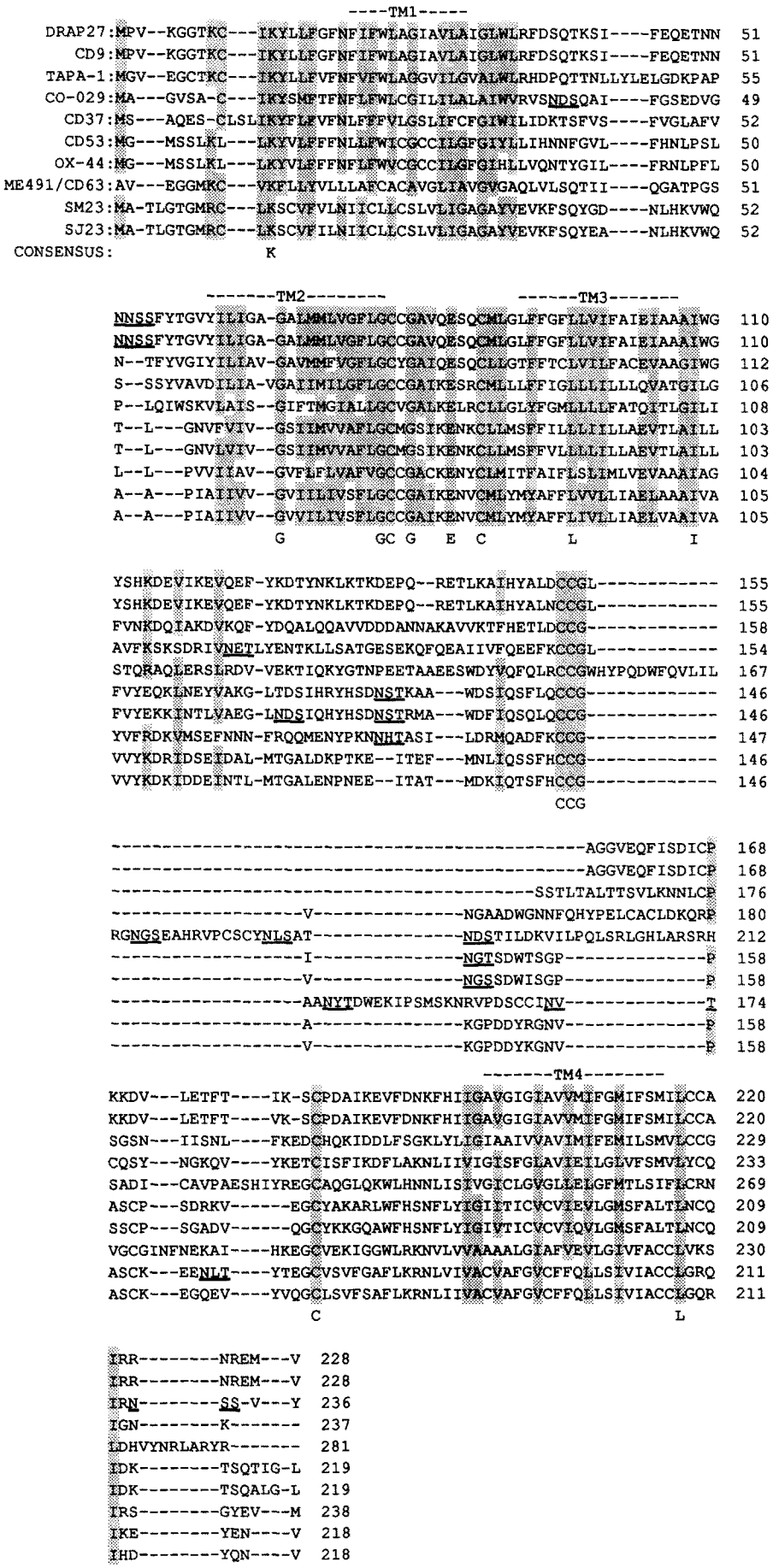

Figure 7. Alignment of the amino acid sequences of DRAP27 and the homologous transmembrane proteins. The amino acid sequences of 10 proteins screened by the method of Pearson and Lipman (49) are aligned through the maximum parsimony method (25). Amino acids conserved in all the proteins are indicated in the bottom line labeled CONSENSUS, and gaps are indicated as −. Identical and physicochemically homologous amino acids found at the same position in more than eight proteins are enclosed in hatched boxes; the combinations of chemically homologous amino acids are as follows: I, L, V, M; F, W, Y; A, G; D, E; H, K, R; S, T; N, Q. TM1, TM2, TM3, TM4 indicate the putative transmembrane domains predicted by Kyte–Doolittle hydropathy analysis with a window width of 15 (33). N-linked glycosylation sites are underlined.

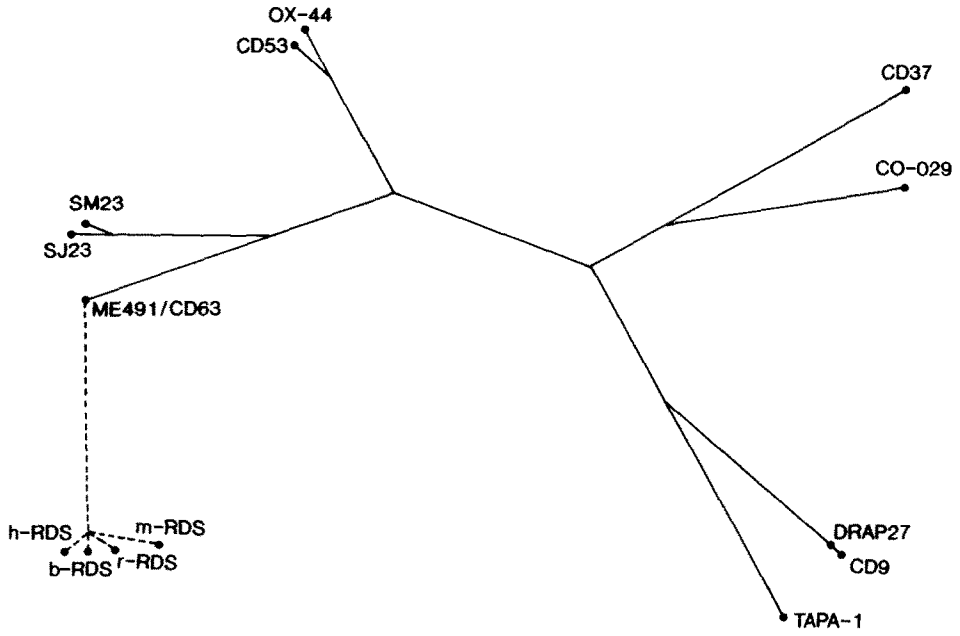

Figure 8. Phylogenic relationship of the amino acid sequences of the new family of transmembrane proteins. The relationship between ME491/CD63 and RDS proteins is indicated by dotted lines, but the distances are not accurate because RDS proteins were not aligned with other proteins. The length of the solid lines corresponds to the relative distances calculated by the maximum parsimony method of Hein (25).

cells? Binding studies with ^{125}I-labeled CRM197 revealed that cells expressing DRAP27 have more DT binding sites than the parental 3279-10 cells. Therefore, we are considering the following possibilities: (*a*) DRAP27 may stabilize the DTR14.5 molecule by associating with it on the cell surface, resulting in an increase in DT receptor molecules on the cell surface, or (*b*) DRAP27 may facilitate the transport of DTR14.5 molecules to the cell surface. Efficient transport would result in an increase in the number of receptors. However, because the Ka value of parental 3279-10 cells for DT could not be determined, we cannot eliminate the possibility that DRAP27 may also modulate the affinity of the DT receptor. Indeed, Scatchard plot analysis showed that C-1 cells, which have fewer DRAP27 molecules than C15-2 cells, had a lower Ka. A change in the affinity of cells to DT might result from association of DRAP27 with DTR14.5, resulting in a conformational change in DTR14.5 that increases affinity. Or, DRAP27 may have a low affinity to DT that cannot be demonstrated in the absence of DTR14.5. When DRAP27 combines with DT receptor, DT may bind more strongly to the complex.

The toxicity of DT for Vero cells is inhibited by some anion transport inhibitors (53), suggesting a role of anion channels in DT intoxication. The structure of DRAP27/CD9 antigen, with four putative transmembrane domains, suggests that this protein may form a channellike structure, as CD20 antigen forms the Ca^{2+} channel (Tedder, T. F., L. J. Zhou, P. D. Bell, R. A. Frizell, and J. K. Bubien. 1990. *J. Cell. Biochem. Suppl.* 14D:195 *Abstr.*; 61). If DRAP27/CD9 antigen forms such a channel, it may be involved in the translocation step of DT as well as the binding step.

What is the physiological function of CD9 antigen? It may play an important role in platelet activation. Most mAbs to CD9 antigen cause the activation of platelets and induce aggregation in the presence of calcium (13, 22, 23, 26, 29, 50). Association of CD9 antigen with the platelet glycoprotein IIb–IIIa complex has been demonstrated (57). Activation of platelets by CD9 antibody is mediated b the Fcγ receptor (66), which may indicate that these molecules are involved in transmembrane signal transduction. CD9 antigen is also expressed in a wide variety of hemopoietic and nonhemopoietic tissues (12). CD9 antigen is expressed on pre-B cells and leukemia cells derived from cells of this stage (30). Although CD9 antigen is not expressed on mature circulating B and T cells, the expression is induced by stimulation with mitogen or alloantigen. CD9 antibody labels various tissues including vascular smooth muscle cells, cardiac muscle cells, and the distal tubules of the kidney. The widespread distribution of CD9 antigen suggests that it plays a fundamental role. The number of molecules of DRAP27/CD9 antigen is much greater than the number of DTR14.5 molecules on Vero cells or human cells (28). This suggests that a large fraction of CD9 antigen is not associated with the DT receptor. CD9 antigen may serve as a positive effector protein in a variety of cell surface receptor systems.

We found a total of 13 related proteins using the homology search procedure of Pearson and Lipman (49). These proteins have a set of common structural motifs, i.e., four transmembrane domains, a relatively long extracellular domain between the third and the fourth transmembrane domains, and no signal sequences. These proteins constitute a new family of transmembrane proteins, of which subsets have been recognized previously (34, 47). The proteins in this family seem to have diverged from a common ancestral protein.

The regions from the first to the third putative transmembrane domains are well conserved among all members of this family except the RDS, suggesting that these domains play important roles in the structure and/or function of these proteins. Although the putative extracellular domains between the third and fourth transmembrane domains are highly divergent, the Cys-Cys-Gly sequences in this region are conserved in all the proteins.

TAPA-1 was initially defined as the target of an antiproliferative antibody in human lymphoid cells, but the antibody reacts with a variety of lymphoid and nonlymphoid human

cell lines (47). An interesting feature of TAPA-1 is that this molecule associates with the 16-kD Leu-13 antigen (60), as DRAP27/CD9 antigen associates with DTR14.5. The wild-type RDS gene is expressed exclusively in photoreceptor cells. It has been proposed to serve as an adhesion molecule for stabilization of the outer segment discs (64).

In conclusion, DRAP27/CD9 antigen associates with the DT receptor. It enhances DT binding activity and sensitivity, at least by increasing the number of DT receptors on the cell surface. As the physiological function of CD9 antigen in cells is not clear, studies on the role of CD9 antigen in the DT receptor system may help to elucidate the general function of CD9 antigen and related proteins.

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