

Cell Surface Monkey CD9 Antigen Is a Coreceptor That Increases Diphtheria Toxin Sensitivity and Diphtheria Toxin Receptor Affinity*

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Jeong-Heon Cha, Joanna S. Brooke, Kathryn N. Ivey, and Leon Eidels‡

From the Department of Microbiology, The University of Texas Southwestern Medical Center, Dallas, Texas 75235-9048

Monkey (Mk) CD9 antigen has been shown previously to increase the diphtheria toxin (DT) sensitivity of cells when co-expressed with Mk proHB-EGF (DT receptor). We have elucidated here the mechanism whereby Mk CD9 influences Mk proHB-EGF and present evidence that Mk CD9 is a coreceptor for DT. We observed that Mk CD9 not only increased the DT sensitivity but also increased the DT receptor affinity of cells. Furthermore, the higher the Mk CD9/Mk proHB-EGF ratio, the higher the affinity. In contrast, mouse (Ms) CD9 did not increase the toxin sensitivity or receptor affinity of cells when co-expressed with Mk proHB-EGF. Using Mk/Ms chimeric CD9 molecules, we determined that the second extracellular domain of Mk CD9 is responsible for both increased sensitivity and receptor affinity. This domain of Mk CD9 also interacts with Mk proHB-EGF in a yeast two-hybrid system. Our findings thus suggest that Mk CD9 has a direct physical interaction with Mk proHB-EGF to form a DT receptor complex and that this contact may change the conformation of the receptor to increase DT binding affinity and consequently increase toxin sensitivity. We thus propose that Mk CD9 is a coreceptor for DT.

Diphtheria toxin (DT)¹ is an exotoxin secreted by lysogenic *Corynebacterium diphtheriae*. The toxin is translated as a single polypeptide (M_r 58,342), and when it is subjected to partial proteolysis it is cleaved into two functionally distinct fragments, an A fragment (M_r 21,167) and a B fragment (M_r 37,195), that remain linked by a disulfide bond (1–4). DT binds through the B fragment to specific eukaryotic cell surface receptors; following binding, receptor-mediated endocytosis enables DT to enter the endosome/lysosome pathway (5). The acidic environment of the endosome causes conformational changes in DT that lead to the translocation and release of the A fragment into the cytosol; the A fragment is enzymatically active and inhibits protein synthesis by ADP-ribosylation of

elongation factor 2. Despite the fact that all elongation factors 2 are susceptible to DT (6), only cells bearing specific DT receptors are DT-sensitive (7).

Our laboratory previously cloned the cDNA encoding a DT receptor from highly toxin-sensitive monkey (Mk) Vero cells (8). The expression of the DT receptor in normally toxin-resistant mouse L-M(TK⁻) cells resulted in DT-sensitive cells (DT^s-II) (8). The predicted amino acid sequence of the DT receptor was found to be identical to that of the cell surface-expressed precursor of heparin-binding EGF-like growth factor (proHB-EGF) (9, 10). The mature/soluble heparin-binding EGF-like growth factor (HB-EGF) is a member of a family of growth factors that includes epidermal growth factor, transforming growth factor α , and amphiregulin (11). Analysis of the amino acid sequence of Mk proHB-EGF revealed a signal sequence (residues 1–23), an extracellular domain (residues 24–159), a transmembrane domain (residues 160–184), and a carboxyl-terminal cytoplasmic domain (residues 185–208) (8, 12). On the cell surface, proHB-EGF may be cleaved and released as a mature/soluble HB-EGF (residues 63–148) (13). The released HB-EGF is able to act as a mitogen by binding to the EGF receptor (9), while the remaining cell surface-bound proHB-EGF is able to function as a juxtacrine growth factor (14) as well as a DT receptor in toxin-sensitive cells (8, 15).

Mekada and colleagues (16) screened monoclonal antibodies raised to Vero cells and identified a monoclonal antibody that protected against DT-mediated cytotoxicity. The antigen (DRAP 27) that corresponded to the monoclonal antibody was cloned and characterized as the monkey homolog of the human CD9 antigen (17). The Mk CD9 antigen alone did not confer DT sensitivity to mouse L-M(TK⁻) cells, but cell surface Mk CD9 expression in a mildly DT-sensitive cell line caused an increased sensitivity to the toxin (17). Our laboratory also showed that the expression of Mk CD9 in the already toxin-sensitive DT^s-II cells made the cells hypersensitive to DT (a 10-fold increase in sensitivity) (18). Therefore, Mk CD9 increases DT sensitivity when co-expressed with Mk proHB-EGF.

CD9 is a member of the tetraspanin superfamily, which is characterized by the presence of four highly conserved hydrophobic transmembrane domains forming two extracellular domains (19, 20) (Fig. 4). The first extracellular domain between transmembrane domains 1 and 2 is small (20–27 amino acids), while the second extracellular domain between transmembrane domains 3 and 4 is larger (75–130 amino acids) (19). Both the amino and carboxyl termini of the tetraspanin superfamily contain short cytoplasmic domains (5–14 amino acids) (19). CD9 is found in virtually all tissues along with other members of the superfamily including CD81, CD82, and CD63 (20). CD9 has been reported to be associated with such biological activities as platelet activation and adhesion, B-cell development, cell metastasis, and motility (19, 20).

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‡ To whom correspondence should be addressed: Dept. of Microbiology, The University of Texas Southwestern Medical Center, 6000 Harry Hines Blvd., Dallas, TX 75235-9048. Tel.: 214-648-5930; Fax: 214-648-5907; E-mail: LEIDEL@MEDNET.SWMED.EDU.

¹ The abbreviations used are: DT, diphtheria toxin; Mk, monkey; EGF, epidermal growth factor; proHB-EGF, heparin-binding EGF-like growth factor precursor; HB-EGF, heparin-binding EGF-like growth factor; Ms, mouse; IC₅₀, the concentration of DT that inhibits protein synthesis by 50% in a cytotoxicity assay; K_d , the apparent dissociation constant of ¹²⁵I-labeled DT from the HB-EGF precursors as measured by a cell surface binding assay; CMV, cytomegalovirus; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

The mechanism by which Mk CD9 increases DT sensitivity of cells is not well understood. It is possible that Mk CD9 increases DT sensitivity by (i) increasing the number of DT receptors through a chaperone-like function and/or protecting them from proteolytic cleavage and/or (ii) increasing DT receptor affinity by a physical interaction on the cell surface that changes the conformation of the receptor.

Previously, Brown *et al.* (18) characterized one cell line (DT^s-III, a mouse L-M(TK⁻) cell line expressing both Mk CD9 and Mk proHB-EGF) and reported that the expression of cell surface Mk CD9 increased DT sensitivity of cells due to an increase in the number of DT-binding sites but apparently did not change DT receptor affinity. Iwamoto *et al.* (15) characterized a similar cell line (also a mouse L-M(TK⁻)-like cell line expressing both Mk CD9 and human proHB-EGF) and reported the same increase in the number of DT-binding sites with no change in DT receptor affinity. Thus, it appeared that Mk CD9 increases DT sensitivity of cells by increasing the number of DT receptors. Recently, however, during our studies on the function of CD9, we observed several L-M(TK⁻) cell lines co-expressing Mk CD9 and Mk proHB-EGF that showed an altered DT receptor affinity. We have now carefully revisited this issue.

In the present study, we have constructed cell lines that express different levels of cell surface Mk CD9 or Mk proHB-EGF, and we have analyzed the influence of these two components on both the DT sensitivity and DT receptor affinity of these cells. In addition, we identified which extracellular domain of Mk CD9 is responsible for the increased toxin receptor affinity and sensitivity of cells as well as for the direct physical interaction with proHB-EGF. We propose a new role of Mk CD9 as a coreceptor for DT.

EXPERIMENTAL PROCEDURES

Materials—The plasmid vectors pcDNA3 and pCDM8, the *Escherichia coli* host strains TOP10F⁺ and DH5 α , and the cDNA Cycle and Original TA Cloning (pCR2.1) kits were purchased from Invitrogen. BALB/c mouse lung poly(A)⁺ RNA was purchased from CLONTECH. [¹²⁵I]NaI (IMS 30; 13–17 mCi/ μ g) and L-[4,5-³H]leucine (60 Ci/mmol) were obtained from Amersham Pharmacia Biotech. Partially purified DT was purchased from Connaught Laboratories (Ontario, Canada), purified further by anion exchange chromatography according to published methods (21) with modifications, and radioiodinated as described elsewhere (22). Polyclonal goat anti-human HB-EGF IgG was from R & D Systems. Monoclonal mouse anti-human CD9 IgG and rat anti-mouse CD9 IgG were from The Binding Site and PharMingen, respectively. Horseradish peroxidase-conjugated secondary antibodies (rabbit anti-goat IgG, goat anti-mouse IgG, and goat anti-rat IgG) were from Jackson ImmunoResearch Laboratories. All other reagents were as described previously (23, 24).

Cell Lines—To examine the effect of cell surface Mk CD9 on DT sensitivity and DT receptor affinity of cells, we used DT^s-II-like cells (L-M(TK⁻) cells that express Mk proHB-EGF) as hosts for the expression of Mk CD9. We reported previously that the original DT^s-II cell line had a high nonspecific binding of radioiodinated DT (over 35%), due to a low cell surface Mk proHB-EGF expression (18). Therefore, it was necessary to construct a new host cell line that has a high level of cell surface Mk proHB-EGF expression and shows a low nonspecific binding of DT. This new host cell line was constructed by transfection of pMkHB-EGF into the normally DT-resistant mouse L-M(TK⁻) cell line and cloned as described previously (24). The cloned cell line stably expressing the highest level of cell surface Mk proHB-EGF was designated as DT^s-II^h and was used throughout this study. DT binding assays with this cell line and with DT^s-II^h transfectants expressing cell surface Mk or mouse (Ms) CD9 showed a low level of nonspecific binding of radioiodinated DT (10–20%).

To examine the influence of cell surface expression of Mk proHB-EGF on DT sensitivity and DT receptor affinity of cells, we used LCD9 cells as hosts. LCD9 cells are mouse L-M(TK⁻) cells that stably express Mk CD9 (DRAP27) cell surface molecules (18). The LCD9 cell line is DT-resistant and hygromycin-resistant; the construction and biochemical characterization of this cell line is described in detail elsewhere

(18). LCD9 cells were transfected with pMkHB-EGF and cloned cell lines stably expressing different levels of Mk proHB-EGF were obtained.

Plasmid Construction—The plasmid pcDNA3 is a mammalian expression vector that allows growth of mammalian cells in the presence of Geneticin. Mk proHB-EGF (8) and Ms proHB-EGF (K133I/H141E) cDNAs (24) were subcloned into pcDNA3 using the *Hind*III and *Xba*I restriction sites as described previously (24). The resulting plasmids were named pMkHB-EGF and pMsHB-EGF(K133I/H141E), respectively.

Previously, to express Mk CD9, cotransfection of pCD9 and pHyg into cells (18) was performed. To improve the transfection efficiency it was necessary to construct a vector that carries both a high expression promoter and a hygromycin resistance selectable marker. pCMV7, a strong mammalian expression vector that carries a CMV promoter, was modified for the present study by adding a hygromycin resistance selectable marker. pCMV7 is a derivative of pCMV5 (25, 26), into which a generic intron consisting of a 5' donor from adenovirus (160-base pair fragment) and a 3' acceptor from an immunoglobulin gene (100-base pair fragment) (27, 28) were ligated into the *Eco*RI and *Pst*I sites of pCMV5. The hygromycin resistance gene was excised from pBabe-Hygro (29), a poly(A) termination sequence was added at its 3'-end, and the fragment (the hygromycin resistance gene with the SV40 intron/pA termination sequence) was introduced downstream of the SV40 *ori* promoter of pCMV7. The resulting plasmid is named pCHA7; the plasmid carries the strong CMV promoter and the hygromycin resistance gene under the control of the SV40 *ori* promoter.

The pCHA7 vector was used for the transfections of DT^s-II^h cells and subsequent expression of Mk CD9, Ms CD9, MsCD9-MkI_{ex}, and MsCD9-MkII_{ex} (Fig. 4). Mk CD9 cDNA was subcloned from pCD9 (18) using *Pst*I and *Bam*HI restriction enzymes into the same restriction sites of pCHA7.

For Ms CD9 cDNA, the first-strand Ms cDNA was generated by reverse transcriptase-PCR (using the cDNA Cycle Kit) from BALB/c mouse lung poly(A)⁺ RNA, and the Ms CD9 was amplified from the Ms cDNA using the primers MsCD9(5') and MsCD9(3') listed in Table I. The PCR fragment was cloned into pCR2.1, resulting in pCR2.1-MsCD9. The fragment was digested with *Xba*I and *Bam*HI restriction enzymes from pCR2.1-MsCD9 and subcloned into the same restriction sites of pCHA7, resulting in pMsCD9. The Ms CD9 gene was confirmed by automated sequencing using the ABI PRISM dye terminator cycle sequencing ready reaction kit (purchased from Perkin-Elmer) with the ABI Prism 377 DNA Sequencer at the sequencing facilities of our institution. This sequence of Ms CD9 reveals three different nucleotides (C363T, T408A, and A447G) from the previously reported sequence of Ms CD9 (30), but these differences did not change the deduced amino acid sequence.

Two chimeric CD9 constructs (MsCD9-MkI_{ex} and MsCD9-MkII_{ex}; Fig. 4) were generated using the PCR technique of gene fusion by overlap extension (31, 32). Five PCRs using six primers listed in Table I were performed to construct each of the CD9 chimeras. The first chimera, MsCD9-MkI_{ex}, was constructed as follows. A DNA fragment (designated as Ms1–34) encoding Ms CD9 amino acid residues 1–34 was PCR-amplified from pMsCD9 with primers MsCD9(5') and ExI-1-down. A second DNA fragment (designated as MkI_{ex}) encoding Mk CD9 amino acid residues 35–58 was PCR-amplified from pMkCD9 with primers ExI-1-up and ExI-2-down. A third DNA fragment (designated as Ms55–226) encoding Ms CD9 amino residues 55–226 was PCR-amplified from pMsCD9 with primers ExI-2-up and MsCD9(3'). The PCR fragments of Ms1–34 and MkI_{ex} were combined and amplified with MsCD9(5') and ExI-2-down, resulting in Ms34MkI_{ex}. The PCR fragments of Ms34MkI_{ex} and Ms55–226 were combined and amplified with MsCD9(5') and MsCD9(3'), resulting in MsCD9-MkI_{ex}. The PCR product (MsCD9-MkI_{ex}) was digested with *Xba*I and *Bam*HI restriction enzymes (these restriction sites were incorporated in the PCR primers) and subcloned into the same sites of pCHA7, resulting in pMsCD9-MkI_{ex}. The sequence of MsCD9-MkI_{ex} insert was confirmed by automated sequencing as described above.

The second chimera, MsCD9-MkII_{ex}, was constructed as follows. A DNA fragment (designated as Ms1–110) encoding Ms CD9 amino acid residues 1–110 was PCR-amplified from pMsCD9 with primers MsCD9(5') and ExII-1-down. A second DNA fragment (designated as MkII_{ex}) encoding Mk CD9 amino acid residues 113–192 was PCR-amplified from pMkCD9 with primers ExII-1-up and ExII-2-down. A third DNA fragment (designated as Ms191–226) encoding Ms CD9 amino residues 191–226 was PCR-amplified from pMsCD9 with primers ExII-2-up and MsCD9(3'). The PCR fragments of MkII_{ex} and Ms191–226 were combined and amplified with ExII-1-up and

TABLE I
Primers used for chimeric CD9 constructs

Boldface italic letters indicate Mk CD9 sequences. Lightface letters indicate Ms CD9 sequences. The restriction enzyme sites for *Xba*I and *Bam*HI are underlined in the sequences of MsCD9(5') and MsCD9(3') primers, respectively. Initiation codon (ATG) letters and the complementary sequence letters (CTA) of termination codon (TAG), in the sequence of the MsCD9(5') and MsCD9(3') primers, respectively, are shown with an overline. Two italic letters in the sequence of MsCD9(5') and MsCD9(3') primers indicate the extra sequence for the *Xba*I and *Bam*HI digestions.

Primers	Primer sequences
MsCD9(5')	GCTCTAGATGCCGGTCAAAGGAGGTAGCAAGTGC
MsCD9(3')	CGGGATCCTAGACCATTTCTCGGCTCCTCGGGATGG
ExI-1-up	CAGTGCTTGCTATTGGACTATGGCTGCGATTTCGACTCTCAGACC
ExI-1-down	GGTCTGAGAGTTCGAATCGCAGCCATAGTCCAATAGCAAGCACTG
ExI-2-up	CAATAAATAATTCAGCTTCTACACAGGAGTGTACATTCTGATTGGAGC
ExI-2-down	GCTCCAATCAGAAATGTACACTCCGTGTAGAAGCTGGAATTATTATTG
ExII-1-up	CCGCCGTCTGGGGCTATACC CACAAGGATGAGGTGATTAAGG
ExII-1-down	CCTTAATCACCTCATCCTTGTGGTATAGCCCCAGACGGCCG
ExII-2-up	CATCAAGAGGCTTTCGACAATAAATCCACATCATTGGAGCAGTGG
ExII-2-down	CCACTGCTCCAATGATGTGGAA TTTATTGTGCAAGACCTCTTTGATG

MsCD9(3'), resulting in MkII_{ex}119Ms. The PCR fragments of MsI-110 and MkII_{ex}119Ms were combined and amplified with MsCD9(5') and MsCD9(3'), resulting in MsCD9-MkII_{ex}. The PCR product (MsCD9-MkII_{ex}) was digested with *Xba*I and *Bam*HI restriction enzymes (these restriction sites were incorporated in the PCR primers) and subcloned into the same sites of pCHA7, resulting in pMsCD9-MkII_{ex}. The insert was confirmed by automated sequencing as described above.

Transfection—Electroporation was used to transfect plasmid DNA into L-M(TK⁻), LCD9, DT^s-II^b, and Ms(K133I/H141E) cells using the BTX ECM 600 system as described previously (24) with the following modifications. To select Geneticin-resistant or hygromycin-resistant cell lines we used a selective medium (Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B, 2 mM L-glutamine, and 1 mg/ml Geneticin or 0.5 mg/ml hygromycin). The cloned proHB-EGF transfectants were screened by the abbreviated cytotoxicity assay described below, and the cloned CD9 transfectants were screened by a quantitative whole cell ELISA described below.

Quantitative Whole Cell ELISAs—To quantify the level of cell surface expression of CD9 and proHB-EGF, quantitative whole cell ELISAs on formalin-fixed cells were performed as described previously (18) with the following modifications. Each cell line was seeded in triplicate wells. Each ELISA included the wild-type Mk proHB-EGF cell line (this cell line expresses both Mk proHB-EGF and Mk CD9 on the cell surface) described previously (24) as a positive control and the L-M(TK⁻) cell line as a negative control.

For the detection of cell surface Mk CD9, the primary antibody employed was a mouse anti-human CD9 IgG, and the secondary antibody was a horseradish peroxidase-conjugated goat anti-mouse IgG. Cell surface Ms CD9 expression was assayed using as primary antibody a rat anti-mouse CD9 IgG and as secondary antibody a horseradish peroxidase-conjugated goat anti-rat IgG. The mouse anti-human CD9 IgG and the rat anti-mouse CD9 IgG recognize the second extracellular domain of Mk and Ms CD9, respectively. The antibodies used to detect Mk CD9 and Ms CD9 on the cell surface did not show any cross-reactivity. Cell surface MsCD9-MkI_{ex} expression was assayed using as primary antibody the rat anti-mouse CD9 IgG and as secondary antibody the horseradish peroxidase-conjugated goat anti-rat IgG. For the detection of cell surface MsCD9-MkII_{ex}, the primary antibody employed was the mouse anti-human CD9 IgG, and the secondary antibody was a horseradish peroxidase-conjugated goat anti-mouse IgG. For the detection of cell surface proHB-EGF, the primary antibody employed was a goat anti-human HB-EGF IgG (which reacts strongly with Mk proHB-EGF and weakly with Ms proHB-EGF), and the secondary antibody was a horseradish peroxidase-conjugated rabbit anti-goat IgG.

Abbreviated Cytotoxicity Assay—We used the previously described abbreviated cytotoxicity assay (23, 24) to observe the inhibition of protein synthesis by DT. Cells were tested with one concentration of DT (1 µg/ml) and no DT as a negative control. Protein synthesis inhibition was measured by [³H]leucine incorporation into acid-precipitable radioactivity as described previously (33, 34). Cell lines in which protein synthesis was inhibited by 50% or more at 1 µg/ml DT were considered DT-sensitive, and those showing less than 50% inhibition were considered DT-resistant. Each assay included the Mk proHB-EGF cell line described previously (24) as a positive control and the LCD9 cell line as a negative control. All individual DT-sensitive cell lines were chosen, subcloned to obtain a pure stable cell population, and reexamined using

the complete cytotoxicity assay, the quantitative whole cell ELISAs for cell surface expression of proHB-EGF and CD9, and the radiolabeled DT binding assay described below.

Cytotoxicity Assay—We used the cytotoxicity assay described previously (24). Cells were tested with DT concentrations of 0, 0.1, 1, 10, 100, and 1000 ng/ml. All cloned DT-sensitive stable cell lines showed at least 80% protein synthesis inhibition at 1 µg/ml DT. All assays were performed in duplicate, and at least two separate assays were performed for each cell line. Each assay included the same positive and negative control cell lines described above for the abbreviated cytotoxicity assay.

Radiolabeled DT Binding Assay—The ability of cell surface Mk or Ms (K133I/H141E) proHB-EGF-expressing cell lines to bind radiolabeled DT was examined as described previously (8, 18). ¹²⁵I-labeled DT (4–400 nM) was added to the cell monolayers with or without a 100-fold excess unlabeled DT. Cell-associated radioactivity was determined employing a Tm Analytical Gamma Trac 1290 γ counting system. Specific binding was determined as the difference between the total cell-associated radioactivity without excess unlabeled DT and the cell-associated radioactivity obtained when a 100-fold excess of unlabeled toxin was included. An acceptable level of nonspecific binding was 20% or less. Specific binding data were subjected to Scatchard analyses. All radiolabeled DT binding assays were performed in duplicate, and at least two assays were performed for each cell line. Each assay included the same positive and negative control cell lines described above for the abbreviated cytotoxicity assay.

Yeast Two-hybrid System—The yeast strain used in these experiments was Y187 purchased from CLONTECH. The first extracellular domain and the second extracellular domain of Mk CD9 were amplified by PCR from pCD9 (18), and the second extracellular domain of Ms CD9 was amplified by PCR from pMSCD9 using the following pairs of oligonucleotide primers (Table II): MkCD9I_{ex}(5') and MkCD9I_{ex}(3'); MkCD9II_{ex}(5') and MkCD9II_{ex}(3'); and MsCD9II_{ex}(5') and MsCD9II_{ex}(3'), respectively. The PCR products were digested with the restriction enzymes *Eco*RI and *Bam*HI and subcloned into the same restriction sites of pGBT9. This resulted in constructs pGBT9/MkCD9I_{ex}, pGBT9/MkCD9II_{ex}, and pGBT9/MsCD9II_{ex}, respectively.

The extracellular domain of the Mk proHB-EGF was amplified by PCR from pMkHB-EGF (24) using primers MkHB-EGF_{ex}(5') and MkHB-EGF_{ex}(3') (Table II). The PCR product (MkHB-EGF_{ex}) was digested with the restriction enzymes *Eco*RI and *Bam*HI and subcloned into the same restriction sites of pGAD424. This resulted in construct pGAD424/MkHB-EGF_{ex}. The extracellular domain of the Ms proHB-EGF was amplified by PCR from pMsHB-EGF (24) using primers MsHB-EGF_{ex}(5') and MsHB-EGF_{ex}(3') (Table II). The PCR product was digested with the restriction enzymes *Eco*RI and *Sa*I and subcloned into the same restriction sites of pGAD424. This resulted in construct pGAD424/MsHB-EGF_{ex}.

Constructs were cotransformed into the Y187 strain using standard procedures suggested by the manufacturer. After growth for 6 days at 30 °C on Trp⁻Leu⁻ drop-out plates, the transformants were tested for β-galactosidase activity using the filter paper protocol provided by the manufacturer. Each assay included pVA3 and pTD1 as positive controls and pGBT9 and pGAD424 (plasmid only) as negative controls. None of the constructs showed the ability to self-activate β-galactosidase transcription.

TABLE II
Primers used for yeast two-hybrid system constructs

EcoRI sites are indicated by underlined italic letters, *Bam*HI sites are underlined, and the *Sal*I site is indicated by a double underline.

Primers	Primer sequences
MkCD9I _{ex} (5')	CTTGCCATCGGAG <u>GAATTC</u> CCTGCGATTTCGAC
MkCD9I _{ex} (3')	GATCAGAATATAGGATCCTGTGTAGAAAGCT
MkCD9II _{ex} (5')	GCCATCTGGGGAG <u>GAATTC</u> CACAAGGATGAG
MkCD9II _{ex} (3')	TGCGCCGATGATGGATCCTTTATTGTCGAA
MsCD9I _{ex} (5')	GCCATCTGGGGAG <u>GAATTC</u> CACAAGGATGAG
MsCD9I _{ex} (3')	CCAATGATGTGGAAGGATCCCTTGTGTGTTGAAGACC
MkHB-EGF _{ex} (5')	TCGGCACTGGTGG <u>GAATTC</u> GAGAGCCTGGAG
MkHB-EGF _{ex} (3')	CACAGCCAGGATGGATCCATGGTCATAGGT
MsHB-EGF _{ex} (5')	CGG <u>GAATTC</u> GAGAGTCTGGAGCGGCTTCGG
MsHB-EGF _{ex} (3')	GGGCGGTCGAGGATCCGTGGTCATATGTATAGGGG

RESULTS AND DISCUSSION

Increasing the Levels of Cell Surface Mk proHB-EGF in LCD9 Cells Results in Cells with Increasing DT Sensitivity but with Decreasing Toxin Receptor Affinity—We investigated the effect of cell surface expression of Mk proHB-EGF on the DT sensitivity and DT receptor affinity of LCD9 cells. LCD9 cells are Ms L-M(TK⁻) cells that stably express cell surface Mk CD9 (DRAP27). Both L-M(TK⁻) and LCD9 cells probably express endogenous cell surface Ms proHB-EGF. However, both of these cell lines are resistant to DT because Ms proHB-EGF does not bind DT (24, 35–37).

Five cloned LCD9 transfectants stably expressing different levels of cell surface Mk proHB-EGF were examined. The levels of cell surface Mk CD9 expression in these cell lines remained constant as assayed by a quantitative whole cell ELISA; there was only ~1.3-fold variation in cell surface Mk CD9 expression among the cell lines. The levels of cell surface Mk proHB-EGF expression varied by ~15-fold as assayed by a quantitative whole cell ELISA; the number of DT-binding sites/cell of these cell lines ranged from 0.5×10^5 (Fig. 1B) as determined by a radiolabeled DT binding assay.

The five cell lines were tested for DT sensitivity (Fig. 1A). The expression of cell surface Mk proHB-EGF in LCD9 cells results in DT-sensitive cell lines. There is a correlation between the level of cell surface Mk proHB-EGF expression and the degree of DT sensitivity; as the number of DT-binding sites expressed in these cell lines increases (from 0.5×10^5 to 1.9×10^6), the level of DT sensitivity of these cell lines also increases (from an IC₅₀ of 7 to 0.4 ng/ml) (Fig. 1).

DT binding assays of these cell lines indicated that as the number of DT-binding sites/cell increases, the DT receptor affinity of the cells decreases (from a K_d of 1.6 nM to 12.3 nM) (Fig. 1B). The cell line with the highest affinity ($K_d = 1.6$ nM) displays $\sim 0.5 \times 10^5$ DT-binding sites/cell (Fig. 1B, ▲) and, as previously reported (18), displays $\sim 1.4 \times 10^7$ cell surface Mk CD9 molecules/cell. Interestingly, the DT receptor affinity of this cell line is similar to that reported for the highly DT-sensitive Mk kidney Vero cells, which have $\sim 1.8 \times 10^4$ DT-binding sites/cell, $\sim 5.6 \times 10^6$ cell surface Mk CD9 molecules/cell, and the highest toxin receptor affinity among cells established in culture ($K_d = 1.1\text{--}2.0$ nM) (7, 18). Furthermore, the ratio of cell surface Mk CD9 to cell surface Mk proHB-EGF of our cell line with the highest affinity is similar to that of Vero cells ($\sim 300:1$) (18). Thus, a large excess of cell surface Mk CD9 over cell surface Mk proHB-EGF appears to be necessary for a cell to possess high affinity for toxin. Since cell surface Mk CD9 also interacts with cell surface Ms proHB-EGF (see below), we cannot ascertain the specific amount of Mk CD9 that is available to interact with cell surface Mk proHB-EGF.

Since increasing the level of cell surface Mk proHB-EGF expression at a constant level of cell surface Mk CD9 decreases the DT receptor affinity of cells, this result suggests that the

ratio of Mk CD9 to Mk proHB-EGF on the cell surface is an important factor in determining toxin receptor affinity. We next tested the reciprocal hypothesis, whether increasing the expression levels of cell surface Mk CD9 at a constant level of cell surface Mk proHB-EGF expression would result in cells with increased affinity for DT.

Increasing the Expression Levels of Cell Surface Mk CD9 on DT^s-II^h Cells Results in Cells with Increasing DT Sensitivity and with Increasing Toxin Receptor Affinity—To analyze the effect of cell surface Mk CD9 levels on DT sensitivity and DT receptor affinity of cells, we employed the DT^s-II^h cell line that expresses a high number of cell surface Mk proHB-EGF in an L-M(TK⁻) cell background. It is important to note that there is no possible interspecies interaction between endogenous Ms CD9 and Mk proHB-EGF, since the wild-type Ms L cell line (17) and our cell lines derived from this cell line do not express endogenous cell surface Ms CD9, as determined by quantitative whole cell ELISAs.

The DT^s-II^h cell line was transfected with pMkCD9, and five cloned cell lines that stably expressed different levels of cell surface Mk CD9 were obtained. The levels of expression of cell surface Mk proHB-EGF in these cell lines were determined by a quantitative whole cell ELISA as well as by an ¹²⁵I-DT binding assay; the levels of cell surface Mk proHB-EGF expression only varied by ~2 fold and by ~1.4 fold, respectively.

The five cell lines were then assayed for DT sensitivity (Fig. 2A). The level of cell surface Mk CD9 expression correlated with the DT sensitivity of these cells; increasing the level of Mk CD9 cell surface expression (from a relative level of 1 to 8.4) resulted in an increased DT sensitivity of cells (from an IC₅₀ of 0.9 to 0.3 ng/ml) (Fig. 2). This represents up to a 10-fold increase in DT sensitivity compared with the host cell line, DT^s-II^h (IC₅₀ = ~3.3 ng/ml). This 10-fold increase in DT sensitivity due to cell surface CD9 expression is consistent with earlier observations (15, 18).

The radiolabeled DT binding assay was used to determine the effect of cell surface Mk CD9 on DT receptor affinity of the five cell lines (Fig. 2B). These cell lines had an increased DT receptor affinity; compared with the DT receptor affinity of the DT^s-II^h host cell line ($K_d = 125$ nM), this increase ranged from ~7- to ~50-fold. From the plot of cell surface CD9 expression level versus K_d (Fig. 2B), it is clear that as the level of cell surface CD9 molecules increased, the DT receptor affinity of these cells also increased (from a K_d of 18.5 nM to 2.5 nM). In contrast to the above described effect of increasing cell surface Mk proHB-EGF expression at a constant cell surface Mk CD9 expression level, increasing the levels of cell surface Mk CD9 in a background of constant cell surface Mk proHB-EGF expression resulted in an increased toxin receptor affinity of these cells.

In summary, it is clear that cell surface Mk CD9 improves the DT receptor affinity. The ratio of Mk CD9 to Mk proHB-

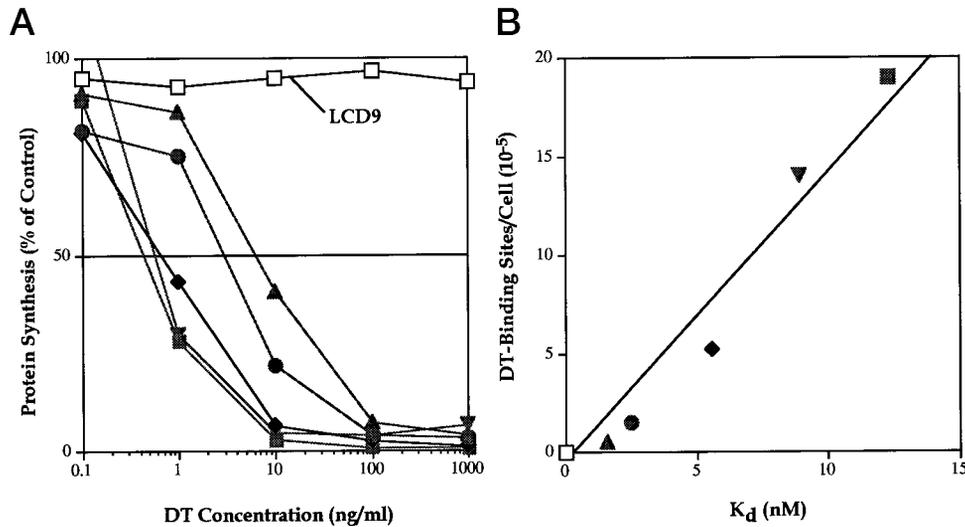


FIG. 1. The effect of the number of cell surface Mk proHB-EGF molecules on DT sensitivity and on DT binding affinity in LCD9 cells. Five cloned transfectants stably expressing different levels of cell surface Mk proHB-EGF in LCD9 cells were examined for DT sensitivity using a cytotoxicity assay and for DT binding using radiolabeled DT. The results shown are from a representative experiment. The level of cell surface Mk proHB-EGF, as determined by a quantitative whole cell ELISA, increased in the following order: \blacktriangle , \bullet , \blacklozenge , \blacktriangledown , \blacksquare . *A*, inhibition of protein synthesis as a function of DT concentration. All assays were done in duplicate and were performed at least twice for each cell line. *B*, cell surface radiolabeled DT binding to cell lines expressing different levels of cell surface Mk proHB-EGF. All assays were done in duplicate and were performed at least twice for each cell line. The number of DT-binding sites/cell was calculated from the cell surface binding data analyzed by the method of Scatchard, and K_d refers to the apparent dissociation constant of ¹²⁵I-labeled DT from the HB-EGF precursors as derived from the same Scatchard plots.

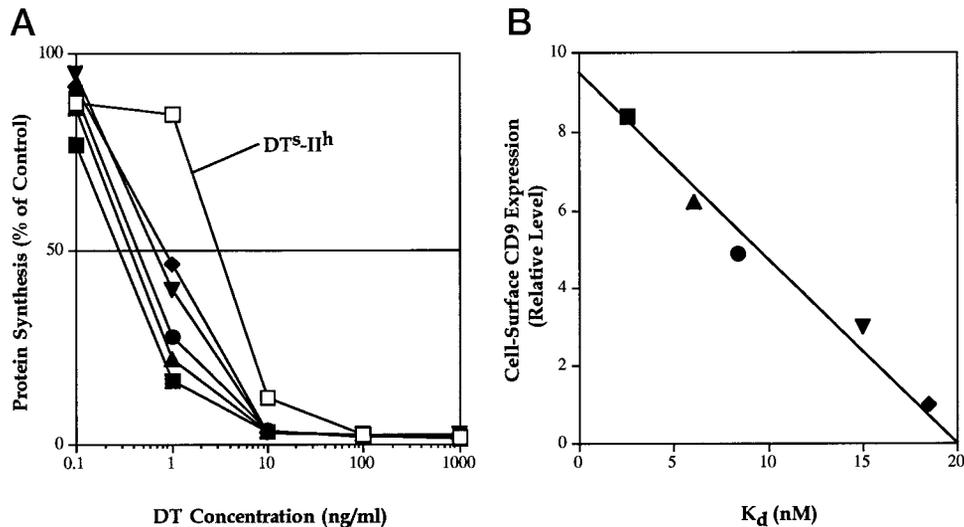


FIG. 2. The effect of the relative level of cell surface Mk CD9 expression on DT sensitivity and on DT binding affinity in DT^s-II^h cells. Five cloned transfectants stably expressing different levels of cell surface Mk CD9 in DT^s-II^h cells were examined for DT sensitivity and DT binding affinity. The abbreviations used and the conditions of the assays were the same as those described in the legend to Fig. 1. CD9 cell surface expression levels were determined by a quantitative whole cell ELISA, and the cell line with the lowest level of expression (\blacklozenge) was set as 1.0; the levels increased in the following order: \blacklozenge , \blacktriangledown , \bullet , \blacktriangle , \blacksquare .

EGF on the cell surface is also important and directly influences the DT sensitivity and receptor affinity of these cell lines. Taken together, these results demonstrate that the higher the ratio of Mk CD9 to Mk proHB-EGF on the cell surface, the higher the toxin sensitivity and receptor affinity. This leads us to suggest that Mk CD9 acts as a coreceptor for DT.

The Second Extracellular Domain of Mk CD9 Contains the DT Coreceptor Function/Activity—Since the expression of Mk CD9 on the cell surface of DT^s-II^h cells results in increased DT sensitivity and DT receptor affinity, we tested whether cell surface Ms CD9 is also able to act as a coreceptor for DT. We found that cell surface expression of Ms CD9 in DT^s-II^h cells does not increase the DT sensitivity or receptor affinity (Fig. 3A and Table III). These results indicate that Ms CD9 is not a coreceptor for DT. Next, exploiting the fact that Ms CD9

changes neither the toxin sensitivity nor the toxin receptor affinity of Mk proHB-EGF-bearing cells, we constructed chimeric CD9 molecules (Fig. 4) in order to identify the domain of the Mk CD9 protein that is responsible for the observed DT coreceptor function.

Transfected cell lines were constructed using the host DT^s-II^h cell line to express cell surface chimeric CD9 molecules that consist of Ms CD9 in which either the first or the second extracellular domain was substituted with the corresponding domain of Mk CD9 as is described in Fig. 4. The stable cell surface expression of chimeric CD9 and of Mk proHB-EGF in the cloned cell lines was verified by quantitative whole cell ELISAs.

The DT sensitivity and receptor affinity increased ~6–8-fold for the DT^s-II^h/MsCD9-MkII_{ex} cell line ($IC_{50} = 0.4$ ng/ml; $K_d =$

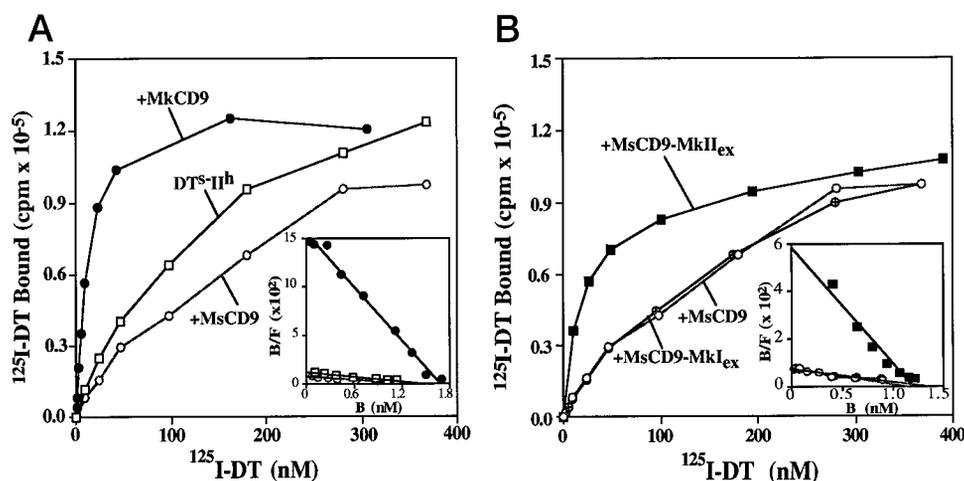


FIG. 3. Radiolabeled DT binding to DT^S-II^h cells and DT^S-II^h cells expressing cell surface Mk CD9, Ms CD9, or chimeric CD9 molecules. Cells were incubated at 4 °C with ¹²⁵I-labeled DT in the absence and in the presence of a 100-fold excess of unlabeled DT in binding medium. After 5 h, the cells were washed with ice-cold phosphate-buffered saline/calcium/magnesium, and the cell-associated radioactivity was assayed. Specific binding was determined by calculating the difference between the total binding with ¹²⁵I-labeled DT and the nonspecific binding obtained with ¹²⁵I-labeled DT in the presence of excess unlabeled DT. The binding data were normalized by cell number/well. All binding assays were done in duplicate and were performed at least twice for each cell line. The results shown are from a representative experiment. Scatchard analyses of the data are presented as insets in each panel. The concentrations of specifically bound ¹²⁵I-labeled DT (referred to as *B*) are plotted on the *abscissa* and the concentrations of bound/free toxin (referred to as *B/F*) are plotted on the *ordinate*. The data were fitted by regression analysis. A, radiolabeled DT binding to DT^S-II^h cell lines without or with cell surface Mk CD9 or Ms CD9. B, radiolabeled DT binding to DT^S-II^h cell lines with cell surface Ms CD9 or chimeric CD9 molecules (MsCD9-MkI_{ex} or MsCD9-MkII_{ex}).

TABLE III

Summary of DT sensitivity, DT binding affinity, and the number of DT-binding sites/cell

n represents the number of DT-binding sites/cell calculated from the cell surface binding data analyzed by the method of Scatchard. All cytotoxicity assays were done in duplicate and were performed at least twice for each cell line, with the average IC₅₀ value being reported ± variation from the mean. All binding assays were done in duplicate and were performed at least twice for each cell line, with the average calculated *K_d* and *n* values ± variation from the mean being reported.

Cell line	IC ₅₀ ng/ml	<i>K_d</i> nM	<i>n</i> × 10 ⁻⁵
DT ^S -II ^h	3.3 ± 1.2	125.0 ± 0.0	9.3 ± 0.7
DT ^S -II ^h /MkCD9	0.4 ± 0.1	10.2 ± 5.8	10.0 ± 0.3
DT ^S -II ^h /MsCD9	3.0 ± 0.8	171.5 ± 40.4	10.5 ± 2.8
DT ^S -II ^h /MsCD9-Mk I _{ex}	2.2 ± 0.7	116.9 ± 36.8	6.1 ± 0.6
DT ^S -II ^h /MsCD9-Mk II _{ex}	0.4 ± 0.1	19.8 ± 0.8	6.4 ± 1.0
Ms(K133I/H141E)	350.0 ± 155.6	ND ^a	ND
Ms(K133I/H141E)/MkCD9	31.0 ± 12.7	61.7 ± 30.6	3.8 ± 1.3
Ms(K133I/H141E)/MsCD9	260.0 ± 42.4	ND	ND
Ms(K133I/H141E)/ MsCD9-Mk I _{ex}	205.0 ± 106.1	ND	ND
Ms(K133I/H141E)/ MsCD9-Mk II _{ex}	32.5 ± 21.9	121.5 ± 30.3	3.4 ± 1.5

^a ND, not detectable because *K_d* and *n* values cannot be measured with cells of very low toxin sensitivity due to high nonspecific binding.

20 nM) as compared with the DT^S-II^h (IC₅₀ = 3.3 ng/ml; *K_d* = 125 nM) and with the DT^S-II^h/MsCD9 cell lines (IC₅₀ = 3 ng/ml; *K_d* = 172 nM) (Fig. 3B and Table III). In contrast, the DT^S-II^h/MsCD9-MkI_{ex} cell line demonstrated a similar DT sensitivity and receptor affinity for toxin (IC₅₀ = 2.2 ng/ml; *K_d* = 117 nM) to that of the DT^S-II^h/MsCD9 cell line (Table III). These data indicate that the coreceptor activity of cell surface Mk CD9 for DT (*i.e.* increased toxin sensitivity and receptor affinity of cells) is contained within its second extracellular domain.

It is interesting to note that the DT-binding ability of the DT^S-II^h/MsCD9-MkII_{ex} cell line is not completely restored to the higher receptor affinity observed for the DT^S-II^h/Mk CD9 cell line (Fig. 3 and Table III). Both of these cell lines, however, express similar levels of CD9 and proHB-EGF on the cell surface. This incomplete reconstitution of DT binding ability for

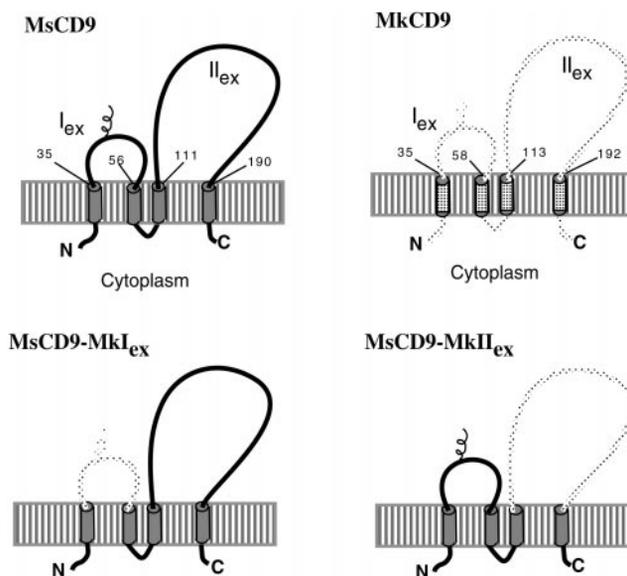


FIG. 4. Chimeric CD9 constructs. The first extracellular domain of Ms CD9 and Mk CD9 extends from Leu³⁵ to Thr⁵⁶ and from Leu³⁵ to Thr⁵⁸, respectively. The second extracellular domain of Ms CD9 and Mk CD9 extends from His¹¹¹ to Lys¹⁹⁰ and from His¹¹³ to Lys¹⁹², respectively. The first extracellular domain of Ms CD9 (Leu³⁵-Thr⁵⁶) was replaced with that of Mk CD9 (Leu³⁵-Thr⁵⁸) to construct a chimeric MsCD9-MkI_{ex} molecule. The second extracellular domain of Ms CD9 (His¹¹¹-Lys¹⁹⁰) was replaced with that of Mk CD9 (His¹¹³-Lys¹⁹²) to construct a chimeric MsCD9-MkII_{ex} molecule. The spiral line indicates the putative glycosylation site on the first extracellular domain.

the DT^S-II^h/MsCD9-MkII_{ex} cell line suggests that the first extracellular domain of Mk CD9 may in fact contribute in a minor way to DT binding.

Since cell surface Ms CD9 is unable to act as a coreceptor for DT when expressed with cell surface Mk proHB-EGF (*i.e.* interspecies interaction), we tested whether it could act as a coreceptor for DT when expressed in a cell line bearing an engineered cell surface Ms proHB-EGF (*i.e.* intraspecies interaction). The host cell line used for this experiment, Ms(K133I/H141E), expresses a mutated Ms proHB-EGF containing only

TABLE IV

Interaction of the extracellular domains of Mk and Ms CD9 antigens with the extracellular domain of Mk and Ms proHB-EGFs employing a yeast two-hybrid system

Filters were incubated on 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside for 4 h at 30 °C. All assays were done at least twice and in duplicate for each set of transformants. Results shown are from a representative experiment. Each assay included pVA3 and pTD1 as positive controls and pGBT9 and pGAD424 as negative controls, provided by Clontech. – indicates no interaction, ++ indicates a moderately strong interaction, and +++ indicates a strong interaction.

pGBT9	pGAD424		
	MkHB-EGF _{ex}	MsHB-EGF _{ex}	pTD1
MkCD9I _{ex}	–	–	–
MkCD9II _{ex}	++	++	–
MsCD9II _{ex}	–	–	–
pVA3	–	–	+++

Mk amino acid residues Ile¹³³ and Glu¹⁴¹, which are sufficient to yield toxin sensitivity (Table III; Ref. 24). The cell surface expression of Mk CD9 in the Ms(K133I/H141E)/MkCD9 cell line resulted in an increased DT sensitivity compared with the host cell line and in the ability to detect specific DT binding (3.8×10^5 DT-binding sites/cell with a K_d of 62 nM) (Table III). Specific DT binding is not detectable in the Ms(K133I/H141E) cell line or in this cell line expressing transfected cell surface Ms CD9 (Table III). These results indicate that cell surface Mk CD9 is a DT coreceptor but that cell surface Ms CD9 is not a coreceptor even when expressed with Ms (K133I/H141E) proHB-EGF. Finally, when the above described cell surface chimeric CD9 molecules (Fig. 4) were expressed in the Ms(K133I/H141E) cell line, only the cell line expressing cell surface MsCD9-MkII_{ex} molecules showed an increased DT sensitivity ($IC_{50} = 33$ ng/ml) and detectable specific DT binding and receptor affinity ($K_d = 122$ nM) compared with the host Ms(K133I/H141E) cell line (Table III). These data provide further evidence that the DT coreceptor function is contained within the second extracellular domain of Mk CD9.

Since Mk CD9 appears to act as a coreceptor for DT, we hypothesized that Mk CD9 and Mk proHB-EGF interact physically. A yeast two-hybrid system was used to test this hypothesis. The expression of β -galactosidase was used as an indicator that the two proteins interact. The first extracellular domain of Mk CD9 does not appear to interact with Mk proHB-EGF, whereas the second extracellular domain of Mk CD9 is able to interact with the extracellular domain of Mk proHB-EGF (Table IV). Interestingly, the second extracellular domain of Mk CD9 is also able to interact with the extracellular domain of Ms proHB-EGF (Table IV). In contrast to Mk CD9, the second extracellular domain of Ms CD9 does not appear to interact with Mk proHB-EGF or with Ms proHB-EGF (Table IV). These results are entirely consistent with those obtained with the coreceptor experiments described above. It is not surprising that the specificity of the physical interaction lies within the second extracellular domain of the CD9 protein; a comparison of the first and second extracellular domains of Mk and Ms CD9 shows that the majority of the amino acid differences are within the second domain (17, 30).

Recently, we reported, using a biosensor method with no other cellular components present, that DT binds to immobilized recombinant human HB-EGF at 25 °C with a K_d of 26.7 nM (38). The affinity of recombinant human HB-EGF for DT observed in this experiment was lower than that reported for radioiodinated DT binding to Vero cells ($K_d = 1.1$ – 2.0 nM) (7, 18). We hypothesized previously that there may be additional factor(s) that participate in the interaction of DT with cell surface Mk proHB-EGF in Vero cells. It is clear from the present study that at least one of these factors is Mk CD9.

Our analyses of the second extracellular domain of Mk CD9 with proHB-EGF, in both the mammalian tissue culture and the yeast systems used in this study, suggest that it is likely that a direct physical interaction between Mk CD9 and proHB-EGF on the cell surface is required for the cell surface Mk CD9 to act as a coreceptor and thus increase toxin sensitivity and receptor affinity. It has been reported that cell surface Mk CD9 expression also results in an increased level of juxtacrine growth factor activity of human proHB-EGF (14). Since the amino acid residues involved in binding DT and those involved in binding to the EGF receptor appear to be located in the same region of Mk proHB-EGF, we speculate that cell surface Mk CD9 may also function as a coligand with cell surface proHB-EGF in binding to the EGF receptor.

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Cell Surface Monkey CD9 Antigen Is a Coreceptor That Increases Diphtheria Toxin Sensitivity and Diphtheria Toxin Receptor Affinity

Jeong-Heon Cha, Joanna S. Brooke, Kathryn N. Ivey and Leon Eidels

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