Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor

Heather M. Scobie, G. Jonah A. Rainey, Kenneth A. Bradley*, and John A. T. Young†

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI 53706

Communicated by R. John Collier, Harvard Medical School, Boston, MA, February 24, 2003 (received for review February 7, 2003)

Bacillus anthracis **secretes two bipartite toxins thought to be involved in anthrax pathogenesis and resulting death of the host. The current model for intoxication is that protective antigen (PA) toxin subunits bind a single group of cell-surface anthrax toxin receptors (ATRs), encoded by the *tumor endothelial marker 8* (*TEM8*) gene. The ATR/TEM8-PA interaction is mediated by the receptor's extracellular domain related to von Willebrand factor type A or integrin inserted domains (VWA/I domains). A metal ion-dependent adhesion site (MIDAS) located within this domain of the ATR/TEM8 protein chelates a divalent cation critical for PA binding. In this report, we identify a second PA receptor encoded by *capillary morphogenesis gene 2* (*CMG2*), which has 60% amino acid identity to ATR/TEM8 within the VWA/I domain, as well as a conserved MIDAS motif. A recombinant CMG2 protein bound PA and mediated toxin internalization when expressed on receptor-deficient cells. Binding between the CMG2 VWA/I domain and PA was shown to be direct and metal-dependent, although the cation specificity of this interaction is different than that observed with ATR/TEM8. Northern blot analysis revealed that CMG2 is widely expressed in human tissues, indicating that this receptor is likely to be relevant for disease pathogenesis. Finally, a soluble version of the CMG2 VWA/I domain inhibited intoxication of cells expressing endogenous toxin receptors when it was added to PA at a 3:1 ratio. These studies distinguish CMG2 as a second anthrax toxin receptor and identify a potent antitoxin that may prove useful for the treatment of anthrax.**

Anthrax is caused by the spore-forming organism *Bacillus anthracis*. Recently, this disease attracted considerable attention as a major bioterrorist threat because the deliberate mailing of *B. anthracis* spores via the U.S. postal system resulted in the deaths of five people (1). This organism secretes two AB-type exotoxins, edema toxin and lethal toxin, that are both thought to play pivotal roles in disease pathogenesis. These toxins share a receptor-binding, B-moiety, protective antigen (PA), but differ in their alternative catalytic A-moieties. The A-moiety of edema toxin, termed edema factor, is a calmodulin-dependent adenylate cyclase that converts intracellular ATP to cAMP. By contrast, lethal toxin contains lethal factor (LF), a zinc-dependent metalloproteinase that cleaves and inactivates most mitogen-activated protein kinase kinases and causes murine macrophage lysis by an unknown mechanism (reviewed in ref. 2).

The first step in intoxication involves binding of an 83-kDa form of PA (PA83) to a cell-surface receptor, where it subsequently undergoes a cleavage by furin to generate a 63-kDa PA subunit (PA63) (3). PA63 spontaneously assembles into a heptameric ring, or prepore, that can bind up to three molecules of either edema factor or LF (4). The oligomerization of PA63 on cell surfaces is associated with receptor clustering and toxin complex internalization (3, 5). After endocytosis, the toxin complex is trafficked to an acidic endosomal compartment where low pH-induced conformational changes in the PA heptamer lead to its insertion in the endosomal membrane and translocation of edema factor and LF into the cytoplasm (6–8).

A human cell-surface receptor, anthrax toxin receptor (ATR), which binds directly to PA and supports cellular intoxication, was recently identified (9). ATR is encoded by the *tumor endothelial marker 8* (*TEM8*) gene, which is expressed in a wide variety of tissues [see National Center for Biotechnology Information (NCBI) UniGene cluster Hs.8966; www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene] but at increased levels in colon tumor vasculature (10). Expression of the mouse homolog of *TEM8* is up-regulated in the vasculature of the developing mouse embryo (11), suggesting that the product(s) of this gene may have some role in neovascularization. However, the physiological function of ATR/TEM8 is not yet known.

At least three different ATR/TEM8 protein isoforms have been described that are produced from alternatively spliced mRNA transcripts (splice variants 1–3, or sv1–3). ATR/TEM8 sv1 and sv2 (GenBank accession nos. NP_115584 and NP_444262, respectively) function as ATRs, whereas the putative secreted sv3 protein (GenBank accession no. NP_060623) does not (refs. 9 and 12; H.M.S. and J.A.T.Y., unpublished data). ATR/TEM8 sv1 and sv2 are both type 1 membrane proteins with the same predicted signal peptide, extracellular region, and putative transmembrane domain. However, these proteins have distinct cytoplasmic tails (that of sv1 is longer by ≈200 aa) that are nonessential for PA binding, toxin uptake, translocation, or intoxication (ref. 9; K.A.B., G.J.A.R., and J.A.T.Y., unpublished data).

The extracellular domains of ATR/TEM8 sv1–3 contain a region that is highly related to von Willebrand factor type A domains (VWA domains), also called integrin inserted domains (I domains). VWA/I domains are well characterized protein interaction sites found in extracellular matrix components or in cell adhesion proteins, like α integrins (reviewed in refs. 13 and 14). Frequently, VWA/I domains contain a metal ion-dependent adhesion site (MIDAS; D*X*D*X*S... T... D, where *X* is any amino acid) that chelates a divalent cation critical for ligand binding (reviewed in ref. 14). The MIDAS motif of ATR/TEM8 was recently shown to be essential for the divalent cation-dependent interaction of this receptor with PA (ref. 9 and unpublished results).

Expression of ATR/TEM8 proteins in a panel of different PA receptor-deficient Chinese hamster ovary (CHO) cell lines restores the sensitivity of these cells to toxin binding (9, 12). Therefore, it has been proposed that these proteins are the only type of anthrax toxin receptor (12). However, in this report we identify the human capillary morphogenesis protein 2 (CMG2) as a second anthrax toxin receptor.

CMG2 is the protein most similar to ATR/TEM8 that has been described to date and has parallel features, which include a signal peptide, type 1 transmembrane region, and a VWA/I domain (15). The two protein sequences share 40% overall amino acid identity and 60% identity within their VWA/I

Abbreviations: PA, protective antigen; LF, lethal factor; VWA, von Willebrand factor type A; I, integrin inserted; EGFP, enhanced GFP; CHO, Chinese hamster ovary; sv, splice variant; DTA, diptheria toxin A chain.

Databank deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY233452).

*Present address: Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, CA 90095.

†To whom correspondence should be addressed. E-mail: young@oncology.wisc.edu.

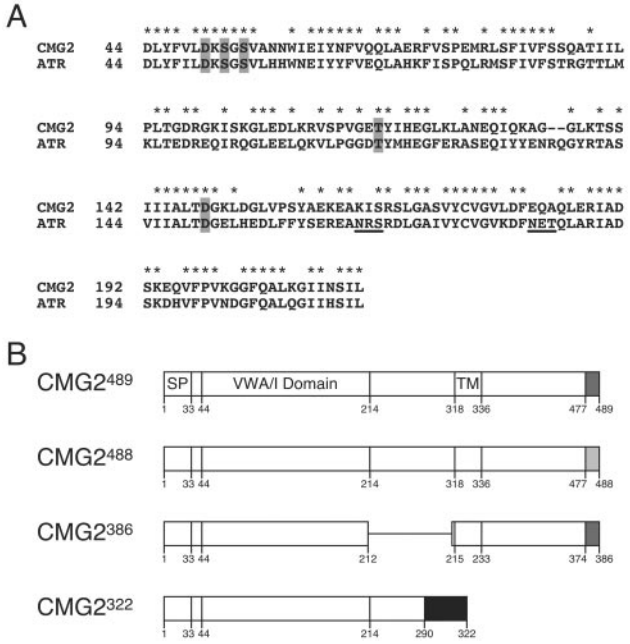

Fig. 1. (*A*) The VWA/I domains of CMG2 and ATR/TEM8 are highly related. The aligned amino acid sequences of the VWA/I domains of each protein (GenBank accession nos. AY233452 and NP_444262, respectively) are shown. Identical residues are indicated by an asterisk, and the five MIDAS motif residues of each protein are indicated by shading. Putative N-linked glycosylation sites found exclusively in ATR/TEM8 are indicated by underlined text. (*B*) Four different CMG2 proteins are identified or predicted to result from six alternatively spliced CMG2 mRNA transcripts. $CMG2^{489}$, described in this article, has a putative signal peptide (SP), extracellular VWA/I domain, and putative transmembrane region (TM). $CMG2^{488}$ (GenBank accession no. BAC03731) is identical to $CMG2^{489}$ except that the last 12 aa of the cytoplasmic tail diverge (indicated by differences in shading). $CMG2^{386}$ (GenBank accession no. AAK77222) is identical to $CMG2^{489}$ except that it lacks amino acid residues 213–315. $CMG2^{322}$ is predicted to be a secreted protein with amino acid residues 1–290, matching those of $CMG2^{488}$ and $CMG2^{489}$ but lacking a transmembrane domain (see NCBI's AceView, locus 118429, sv 4 (g).

domains. *CMG2* was originally identified as a gene expressed at elevated levels in human umbilical vein endothelial cells that were induced to undergo capillary formation in three-dimensional collagen matrices (15). Although the precise function of CMG2 is not yet known, its VWA/I domain binds selectively to collagen type IV and laminin, suggesting that these are its natural ligands *in vivo* (15). After using RT-PCR analysis, it was concluded that *CMG2* gene expression is restricted to human placental tissue (15). However, EST data available from NCBI indicate that this gene is expressed in a wide variety of different tissue types (see NCBI UniGene cluster Hs.5897).

Several different CMG2 protein isoforms, encoded by alternatively spliced mRNA transcripts, have been identified or predicted (Fig. 1). The previously described 386-aa protein is hereafter referred to as $CMG2^{386}$ (15). It has been concluded from fluorescence microscopy studies that the $CMG2^{386}$ isoform is expressed predominantly within the endoplasmic reticulum of cells and not at the plasma membrane (15). In addition, there is an uncharacterized 488-aa isoform of CMG2 ($CMG2^{488}$) that differs from $CMG2^{386}$ in that it includes a 100-aa membrane-proximal region between the VWA/I domain and transmembrane region and contains 12 alternative amino acids at its C-terminal end. NCBI's ACEMBLY program, which aligns current mRNA and EST data to genomic DNA sequence to predict all possible gene transcript models, indicates that there are two other potential CMG2 protein isoforms that are 322 and 489 aa in length. $CMG2^{322}$ is predicted to be a secreted protein without a transmembrane domain, whereas $CMG2^{489}$ is identical to $CMG2^{488}$ except that its C-terminal 13 aa match those of $CMG2^{386}$ (see NCBI's AceView locus 118429; www.ncbi.nlm.nih.gov/IEB/Research/Acembly/).

In this article, we have tested whether CMG2 is sufficiently similar to ATR/TEM8 to function as an anthrax toxin receptor. We show that a CMG2 protein is capable of binding PA in a divalent cation-dependent manner and of supporting intoxication when expressed in a PA receptor-deficient rodent cell line. We also show that a soluble version of the CMG2 VWA/I domain effectively blocks intoxication of cultured cells. These data show that CMG2 can function as an anthrax toxin receptor and have led to the discovery of a new and potent anthrax antitoxin.

Materials and Methods

DNA Constructs. A 3′ SMART RACE RT-PCR amplification protocol (CLONTECH) was used to generate cDNA pools from human placental mRNA (CLONTECH). The $CMG2^{489}$ ORF (GenBank accession no. AY233452) was isolated multiple times by using nested PCR with CMG2 (GenBank accession no. AY040326) gene-specific oligonucleotide primer sets: 5′-GCCACCTTTGCGACCCTCCTGAGCTTAGG-3′ and 5′-TATTTCCCTGCCTCCATTATACTGACTCAAGCAG-3′; 5′-aactcgagAGGATGGTGGCGGAGCGGTC-3′ and 5′-attagatctccAGCAGTTAGCTCTTTCTCAATACATTCCC-3′ [CMG2 sequence is indicated with capital letters; restriction sites used to generate the enhanced GFP (EGFP) fusion gene in the murine leukemia virus-based retroviral vector pLEGFP.N1 (CLONTECH) are underlined]. An ATR/TEM8 sv2-EGFP fusion gene was also cloned in the pLEGFP.N1 and encodes a protein with amino acid residues 1–368 of ATR/TEM8 (GenBank accession no. NM_053034) fused at the *Bam*HI site with EGFP coding sequence. Retroviral vectors were produced by using a previously described transient transfection protocol (9). A gene encoding $CMG2^{VWA/I}$-MycHis with amino acids 1–232 of $CMG2^{489}$ was PCR amplified and fused to the MycHis tag in the pcDNA3.1/*myc*-His(−) A vector (Invitrogen) at the *Hind*III site. All constructs were validated by DNA sequence analysis.

Cell Lines. PA receptor-deficient CHO-R1.1 cells derived from CHO-K1 cells were described elsewhere (9). CHO-R1.1 cells were infected with retroviral vectors that encoded either $CMG2^{489}$-EGFP or ATR/TEM8 sv2-EGFP and neomycin phosphotransferase. The transduced cells were then selected in medium containing 900 μg/ml G418, and EGFP-expressing cells were subsequently isolated by flow-cytometric sorting.

Northern Blot Analysis. Northern blot analysis was performed by using a human 12-lane multiple-tissue Northern blot according to the manufacturer's instructions (CLONTECH). The probe used was a 1.5-kb *Age*I/*Xho*I fragment of pLEGFP.N1-$CMG2^{489}$ (corresponding to the full $CMG2^{489}$ ORF) labeled by random hexamer priming with [^{32}P]dCTP (Perkin–Elmer). As a control, the blot was also probed with a human β-actin probe (CLONTECH). After hybridization, the blot was washed for 30 min at 68°C with 0.5× sodium chloride/sodium citrate, 0.1% SDS solution.

Purification of $CMG2^{VWA/I}$-MycHis. Forty 10-cm plates of HEK293 cells were transfected with 10 μg each of the plasmid vector encoding $CMG2^{VWA/I}$-MycHis by calcium phosphate precipitation. Medium was collected 48, 72, and 90 h later, pooled, and purified on an Ni-NTA column (Qiagen, Valencia, CA), and the $CMG2^{VWA/I}$-MycHis protein was eluted with 40 mM imidazole. This fraction was dialyzed against Tris-buffered saline (TBS),

quantified by using a protein microassay (Bio-Rad), and subjected to electrophoresis on a 12.5% polyacrylamide gel containing SDS, followed by Coomassie blue staining.

PA-Binding Studies. PA binding to CHO-R1.1 cells that expressed $CMG2^{489}$-EGFP or ATR/TEM8 sv2-EGFP was assessed by flow-cytometric analysis; 10^6 cells of each type were incubated with 50 nM PA (1 h, 4°C), washed with PBS, incubated with a polyclonal PA-specific rabbit antiserum (9) (1:500 dilution, 1 h, 4°C), washed with PBS again, and then incubated with an allophycocyanin-conjugated anti-rabbit antibody (Molecular Probes; 1:2,000 dilution, 1 h, 4°C). ELISAs were performed by binding 0.3 μg of PA83 (List Biological Laboratories, Campbell, CA)/100 μl of TBS in a well of a MaxiSorp plate (Nalge). Samples were then treated with TBST solution (TBS containing 3% BSA and 0.05% Tween 20), washed with TBST, and incubated with 0–300 ng of purified $CMG2^{VWA/I}$-MycHis/100 μl TBST in the absence or presence of 1 mM $MgCl_2$, $MnCl_2$, $CaCl_2$, or $ZnCl_2$. The samples were then washed with TBST and incubated with a horseradish peroxidase-conjugated anti-His antibody (1:2,000 dilution; Santa Cruz Biotechnology). All incubations for ELISAs were performed for 1 h at room temperature. The levels of bound antibody were then measured by using Supersignal ELISA Pico chemiluminescent substrate (Pierce) and a luminometer (Victor V, Wallac).

Intoxication Assays. Cell viability assays (WST-1 assay; Roche Molecular Biochemicals) were performed in triplicate by incubating 5,000 cells of each type for 30 h with 2×10^{-10} M LF_N-diptheria toxin A chain (DTA) and varying concentrations of PA (10^{-12} to 10^{-8} M; List Biological Laboratories) or no PA (100% viability control). The inhibition assays were performed as above with different amounts of $CMG2^{VWA/I}$-MycHis (0–1,000 ng/100 μl) added to 10^{-9} M PA and 10^{-10} M LF_N-DTA before this mixture was added to cells. The IC_{50} was determined by regression analysis (PRISM, GraphPad, San Diego).

Results

CMG2 Protein Binds PA in a Divalent Cation-Dependent Manner. The VWA/I domain of ATR/TEM8 serves as the site for PA binding (9) and is 60% identical to the VWA/I domain of CMG2 (Fig. 1*A*). To test whether CMG2 can bind PA, an RT-PCR-based approach was used that led to the isolation of the $CMG2^{489}$ ORF from human placental mRNA (GenBank accession no. AY233452; Fig. 1*B*). We then constructed a recombinant $CMG2^{489}$-EGFP gene that encodes this CMG2 isoform fused to the EGFP to facilitate protein detection.

To test whether $CMG2^{489}$-EGFP was localized to the cell surface and could bind PA, this protein was expressed in a stably transduced population of PA receptor-deficient CHO-R1.1 cells. PA binding was assayed by flow cytometry after incubating these cells with PA, followed by anti-PA serum and an allophycocyanin-conjugated secondary antibody. For control purposes, these experiments were also performed with the parental receptor-deficient CHO-R1.1 cells, CHO-R1.1 cells that expressed a similar ATR/TEM8 sv2-EGFP fusion protein, and wild-type CHO-K1 cells that express endogenous PA receptors. These studies revealed that cells expressing either the CMG2 or ATR/TEM8 fusion protein were equally competent for PA binding (Fig. 2*A*). Thus, we concluded that $CMG2^{489}$-EGFP is expressed at the cell surface and can bind PA.

To investigate whether the CMG2 VWA/I domain, like that of ATR/TEM8, is capable of binding directly to PA in a divalent cation-dependent manner, a soluble $CMG2^{VWA/I}$-MycHis protein was generated. This protein, which consists of amino acids 1–232 of $CMG2^{489}$ fused to a C-terminal MycHis tag, was produced in the supernatants of cultured HEK293 cells and was purified by nickel affinity chromatography (see Fig. 4 *Inset*). This protein was tested in an ELISA for its ability to bind PA, which had been immobilized on wells of a microtiter plate, in the absence or presence of 1 mM $MgCl_2$, $MnCl_2$, $CaCl_2$, or $ZnCl_2$. These experiments showed that PA bound to CMG2 VWA/I domain in a divalent cation-dependent manner with the following cation preference: $Ca^{2+} > Mn^{2+} > Mg^{2+} > Zn^{2+}$ (Fig. 2*B*). Calcium-specific binding distinguishes this PA interaction from that of the ATR/TEM8 VWA/I domain, which does not occur in the presence of this ion (K.A.B., G.J.A.R., and J.A.T.Y., unpublished results).

CMG2 Protein Supports Intoxication. To assess whether $CMG2^{489}$-EGFP could support PA internalization and intoxication, CHO-R1.1 cells expressing this protein were incubated with different amounts of PA and a constant amount of LF_N-DTA. LF_N-DTA is a recombinant protein with the N-terminal PA-binding region of LF fused to the diphtheria toxin catalytic A chain (16). Cell viability after intoxication was measured by using a commercially available assay (WST-1 assay; Roche Molecular Biochemicals). Cells that expressed $CMG2^{489}$-EGFP or ATR/TEM8 sv2-EGFP were killed by the toxin at equivalent levels (Fig. 2*C*). The level of killing observed with these cells was higher than that seen with CHO-K1 cells (Fig. 2*C*), presumably as a result of higher levels of PA-receptor expression (Fig. 2*A*). These data demonstrate that the $CMG2^{489}$ protein functions as an anthrax toxin receptor.

CMG2 mRNA Transcripts Are Readily Detectable in a Number of Different Human Tissue Types. There is conflicting information available on the tissue-specific expression pattern of the *CMG2* gene as discussed previously. To address this controversy, a Northern blot containing 1 μg of poly(A)$^+$ mRNA samples from 12 human tissues (CLONTECH) was hybridized with a radioactively labeled CMG2-specific DNA probe. This analysis revealed three predominant CMG2 mRNA transcripts, estimated to be ≈5.1, 3.9, and 3.4 kb in length, with at least one transcript expressed in 10 of the 12 tissues tested (Fig. 3; transcript lengths were determined by using LABWORKS analysis software, Ultraviolet Products, Upland, CA). *CMG2* expression was detected in heart, lung, liver, skeletal muscle, peripheral blood leukocyte, placenta, small intestine, kidney, colon, and spleen but not in brain and thymus (Fig. 3). Although CMG2 mRNA transcripts have been previously reported in brain tissue (see NCBI UniGene cluster Hs.5897), their level is presumably too low to be detected by Northern blot analysis. The observed CMG2 transcripts corresponded in length to those predicted by NCBI's ACEMBLY method to encode $CMG2^{488}$ (5.23 kb), $CMG2^{322}$ (3.53 kb), and a CMG2 protein unlike the other isoforms in that it is missing the signal peptide, VWA/I domain, and most of the extracellular region (4.06 kb) (see NCBI's AceView locus 118429). Whether the observed transcripts actually encode these proteins or some other CMG2 isoform and whether the proteins predicted are actually produced have yet to be proven experimentally. Nevertheless, these data show that the *CMG2* gene is expressed at readily detectable levels in a variety of different human tissue types.

Soluble CMG2 VWA/I Domain Is a Potent Antitoxin. Previously, it was shown that soluble ATR/TEM8 VWA/I domain protein is an antitoxin that can protect cultured cells from intoxication (9). To test whether a soluble version of the CMG2 VWA/I domain can function similarly, the $CMG2^{VWA/I}$-MycHis protein was tested for its ability to block intoxication of CHO-K1 cells by PA and LF_N-DTA. Strikingly, 100% protection of these cells was achieved at concentrations of ≥300 ng/ml, and 25% protection was observed at a protein concentration of 100 ng/ml (Fig. 4). Based on the 60% representation of $CMG2^{VWA/I}$-MycHis protein in the preparation (Fig. 4 *Inset*; LABWORKS analysis software, Ultraviolet Products) and on its predicted molecular mass calculated by amino acid composition (24.6 kDa), the IC_{50} of this protein is estimated to be 3.1 ± 0.2 nM (Fig. 4).

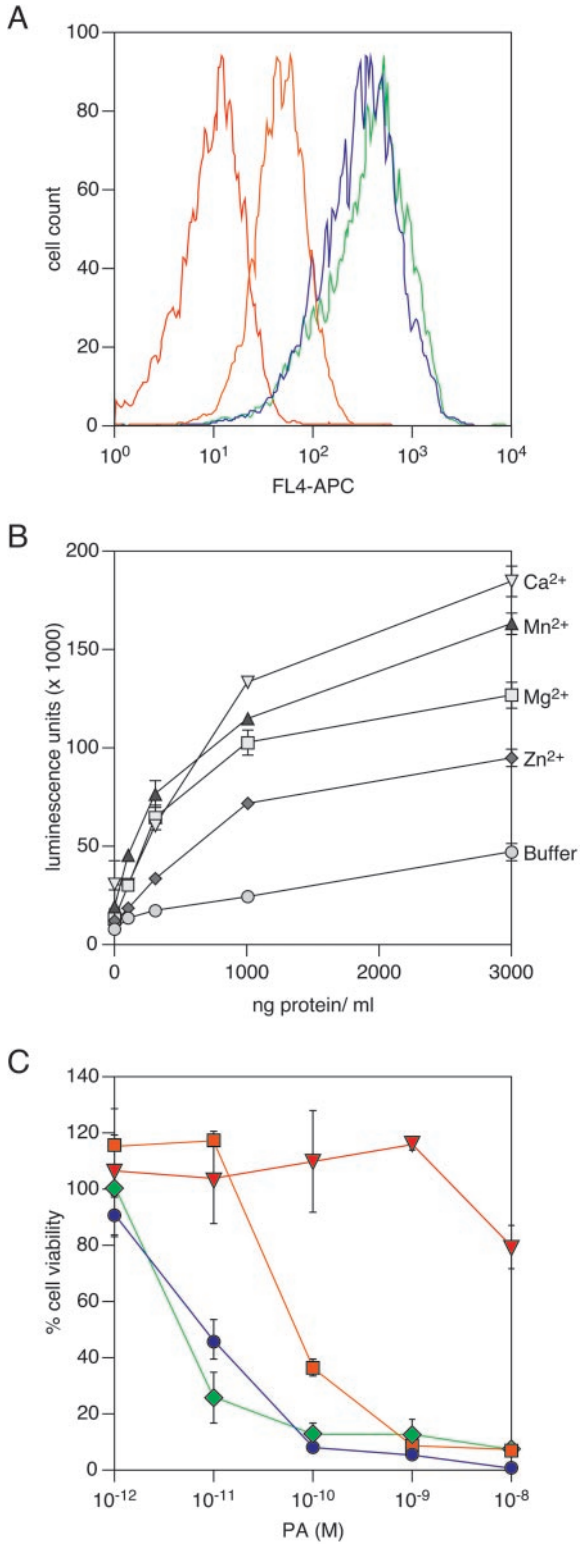

Fig. 2. CMG2 binds PA in a divalent cation-dependent manner and supports intoxication. (*A*) PA receptor-deficient CHO-R1.1 cells expressing $CMG2^{489}$-EGFP (blue line) were incubated with PA, a PA-specific antiserum, and an allophycocyanin-conjugated secondary antibody and then analyzed by flow cytometry. For control purposes, these experiments were also performed with receptor-negative parental CHO-R1.1 cells (red line), CHO-R1.1 cells expressing ATR/TEM8 sv2-EGFP (green line), and wild-type CHO-K1 cells that express endogenous receptors (orange line). (*B*) PA was bound to wells of a microtiter plate and then incubated with varying amounts of purified $CMG2^{VWA/I}$-MycHis protein in the absence or presence of 1 mM $CaCl_2$, $MnCl_2$, $MgCl_2$, or $ZnCl_2$. The bound CMG2 protein was then detected by adding a horseradish peroxidase-conjugated anti-His antibody and a chemiluminescent substrate. (*C*) CHO-R1.1 cells expressing $CMG2^{489}$-EGFP (blue circles) were incubated with a constant amount of LF_N-DTA (2×10^{-10} M) and with increasing amounts of PA (shown). The level of toxin-mediated killing was measured by using a cell viability assay (WST-1 assay; Roche Molecular Biochemicals). The assay was performed in triplicate (standard deviations of the data are indicated with error bars), and the results were normalized to those obtained from cells treated with LF_N-DTA in the absence of PA (100% viable). For control purposes, these experiments were also performed with parental CHO-R1.1 cells (red triangles), CHO-R1.1 cells expressing ATR/TEM8 sv2-EGFP (green diamonds), and CHO-K1 cells (orange squares).

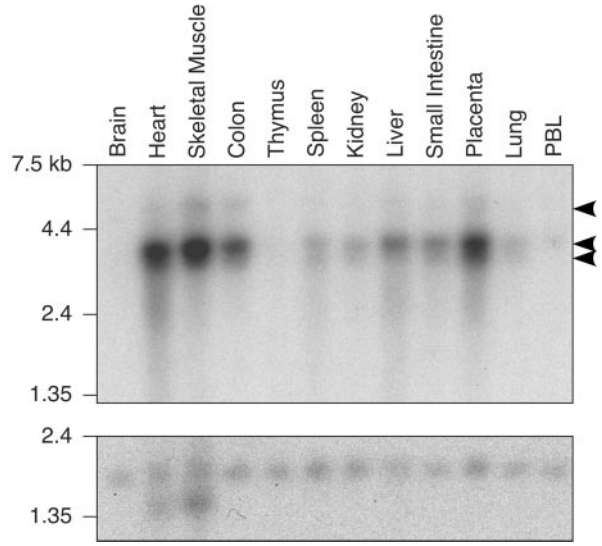

Fig. 3. CMG2 is widely expressed in human tissues. (*Upper*) A radiolabeled CMG2-specific DNA probe was hybridized to a 12-tissue Northern blot (CLONTECH) that contained 1 μg of poly(A)$^+$ mRNA per lane. Three predominant CMG2 mRNA transcripts were detected (indicated by arrowheads) that correspond in size to the 5.23-, 4.06-, and 3.53-kb transcripts predicted by NCBI's ACEMBLY method (see locus 118429). (*Lower*) A radiolabeled β-actin control probe (CLONTECH) was hybridized to the same blot, and it detected the expected 2-kb mRNA transcript in all tissues as well as the expected 1.6- to 1.8-kb transcript variant in heart and skeletal muscle (17).

Discussion

In this article, we demonstrate that an EGFP-tagged form of $CMG2^{489}$ is expressed at the surface of PA receptor-deficient CHO cells, binds PA, and supports intoxication of these cells by PA and LF_N-DTA at levels comparable with that of ATR/TEM8 sv2 expressed in the same context. The cell-surface localization of this form of CMG2 contrasts with that of another CMG2 isoform ($CMG2^{386}$), which is restricted to the endoplasmic reticulum (15). The binding interaction between a soluble version of the CMG2 VWA/I domain and PA depends on the addition of divalent cations. We also show that the soluble CMG2 VWA/I domain functions as a potent antitoxin to protect cultured CHO-K1 cells from intoxication by PA and LF_N-DTA, presumably by acting as a receptor decoy to prevent PA from binding to cell-surface receptors. In these experiments, the calculated IC_{50} of the CMG2 VWA/I domain is 3.1 nM, which corresponds to effective blocking activity at a 3:1 ratio of inhibitor to PA. Taken together, these data provide compelling evidence that CMG2 is a second anthrax toxin receptor.

The divalent cation specificity of the PA–CMG2 interaction suggests that the MIDAS motif of the receptor is involved in binding PA, although this remains to be formally tested by mutagenesis studies. The MIDAS motif of ATR/TEM8 has been shown to be

CELL BIOLOGY

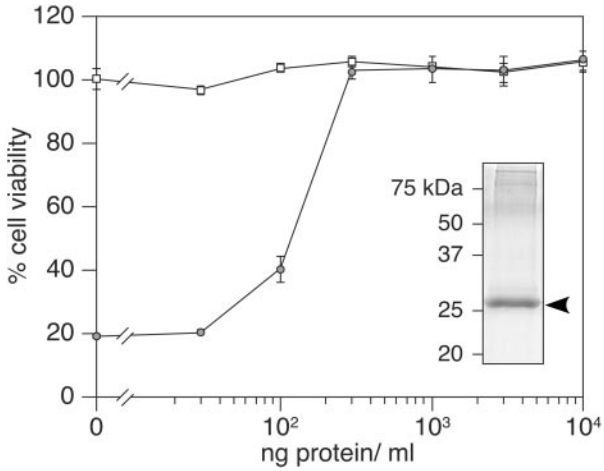

Fig. 4. Soluble CMG2 VWA/I domain protein inhibits intoxication of CHO-K1 cells. CHO-K1 cells, which express endogenous anthrax toxin receptors (●), were incubated with 10^{-9} M PA and with 10^{-10} M LF_N-DTA in the presence of increasing amounts of $CMG2^{VWA/I}$-MycHis protein (0–10 μg/ml). Cell viability after intoxication was then measured (as in Fig. 2) and is shown as a percentage of that obtained with cells treated with PA alone (100% viable). For control purposes, the same experiments were performed with PA receptor-deficient CHO-R1.1 cells (□). (*Inset*) Coomassie blue-stained gel of the purified $CMG2^{VWA/I}$-MycHis protein preparation (indicated by an arrowhead).

important for this interaction, as site-specific mutagenesis of the first residue of this motif (Asp-50) abrogates PA binding and intoxication (K.A.B., G.J.A.R., and J.A.T.Y., unpublished results). However, despite the similarities between these two receptors, they seem to have distinct cation requirements for supporting the PA interaction. In the case of the ATR/TEM8 VWA/I domain, Mn^{2+} functions well to support PA binding, whereas Mg^{2+} functions at an intermediate level and Ca^{2+} does not support binding (K.A.B., G.J.A.R., and J.A.T.Y., unpublished results). When CMG2 was subjected to a similar analysis, the highest level of binding was observed in the presence of Ca^{2+} and Mn^{2+}, and an intermediate level was observed in the presence of Mg^{2+}. Although these data indicate that there are likely to be subtle differences between the PA–ATR/TEM8 and PA–CMG2 interactions, it remains to be seen which of these metal ions is most physiologically relevant in either case.

The data contained in this article confirm that the *CMG2* gene is expressed as three predominant mRNA transcripts in 10 of 12 different human tissues tested, including heart, skeletal muscle, and lung. This expression pattern correlates with EST data compiled by NCBI (see NCBI UniGene cluster Hs.5897) but contrasts with the previously reported RT-PCR expression analysis that indicated that expression of this gene was restricted to human placenta (15). Northern blot analysis detected transcripts that corresponded in length to the transcript models predicted by NCBI's ACEMBLY to encode $CMG2^{488}$, $CMG2^{322}$, and a CMG2 protein missing most of the extracellular region. The analysis did not detect the transcript predicted to encode the $CMG2^{489}$ isoform described in this report (2.08 kb) or two other predicted CMG2 mRNA transcripts encoding $CMG2^{488}$ (4.59 kb) and $CMG2^{386}$ (the originally identified CMG2 clone; 1.77 kb) (see NCBI's AceView locus 118429). It is presently unclear whether the latter transcripts are absent from the tissues tested or whether they are expressed at a level undetectable by this form of analysis. Indeed, the 2.08-kb mRNA transcript predicted to encode $CMG2^{489}$ was not readily detected in the human tissues (including placenta) but was readily isolated multiple times by RT-PCR amplification from human placental mRNA (this article). These data might suggest that the $CMG2^{489}$ protein is encoded by a rare mRNA transcript. However, because ACEMBLY's mRNA and protein predictions of CMG2 gene products are merely based on short EST information at this time, it is also a formal possibility that the $CMG2^{489}$ ORF may be encoded by more than one mRNA transcript, like $CMG2^{488}$, or that one of the transcripts detected by Northern blot may encode $CMG2^{489}$. This possibility can be rigorously examined only after the mRNA profiles of this gene have been more thoroughly characterized. In addition, future studies are needed to determine the relative abundance of each of the CMG2 proteins in different human cell types, as protein levels are affected by additional factors such as protein stability.

The $CMG2^{488}$ protein predicted to be encoded by one of the observed predominant CMG2 mRNA transcripts (5.23 kb) is also expected to serve as an anthrax toxin receptor. This isoform differs from $CMG2^{489}$ only in its C-terminal 12 aa and is likely to be functional because the cytoplasmic tails of ATR/TEM8 proteins are not important for receptor activity (12). Although $CMG2^{386}$ seems to have a complete VWA/I domain, it may not function as an anthrax toxin receptor because it has been observed to be localized intracellularly (15). Based on experiments with ATR/TEM8, neither of the CMG2 proteins predicted to be encoded by the 4.06- or the 3.53-kb transcripts (the protein without a VWA/I domain or the secreted $CMG2^{322}$, respectively) would be predicted to function as a toxin receptor (9, 12).

The *CMG2* and *TEM8* genes are both expressed in a diverse range of different human tissue types (Fig. 3; ref. 9). Therefore, it seems highly likely that both types of receptor are relevant for anthrax pathogenesis. With this in mind, it is not yet clear whether lack of expression of one or both of these proteins accounts for the PA receptor-deficient phenotype of certain mutagenized CHO cell lines (9, 12). Future studies will determine the relative contribution of each receptor to the disease state.

We thank John Naughton, Richard Barnard, and Daniel Knauss for help in preparing this manuscript and Tracie Gibson for technical advice. This work was supported by National Institute of Allergy and Infectious Diseases Grant RO1AI48489. G.J.A.R. is supported by Training in Oncology Grant T32 CA09075.

1. Inglesby, T. V., O'Toole, T., Henderson, D. A., Bartlett, J. G., Ascher, M. S., Eitzen, E., Friedlander, A. M., Gerberding, J., Hauer, J., Hughes, J., *et al.* (2002) *J. Am. Med. Assoc.* **287,** 2236–2252.
2. Mock, M. & Fouet, A. (2001) *Annu. Rev. Microbiol.* **55,** 647–671.
3. Beauregard, K. E., Collier, R. J. & Swanson, J. A. (2000) *Cell. Microbiol.* **2,** 251–258.
4. Mogridge, J., Cunningham, K. & Collier, R. J. (2002) *Biochemistry* **41,** 1079–1082.
5. Abrami, L., Liu, S., Cosson, P., Leppla, S. H. & Van Der Goot, F. G. (2003) *J. Cell Biol.* **60,** 321–328.
6. Benson, E. L., Huynh, P. D., Finkelstein, A. & Collier, R. J. (1998) *Biochemistry* **37,** 3941–3948.
7. Friedlander, A. M. (1986) *J. Biol. Chem.* **261,** 7123–7126.
8. Petosa, C., Collier, R. J., Klimpel, K. R., Leppla, S. H. & Liddington, R. C. (1997) *Nature* **385,** 833–838.
9. Bradley, K. A., Mogridge, J., Mourez, M., Collier, R. J. & Young, J. A. (2001) *Nature* **414,** 225–229.
10. St Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K. E., Montgomery, E., Lal, A., Riggins, G. J., Lengauer, C., Vogelstein, B. & Kinzler, K. W. (2000) *Science* **289,** 1197–1202.
11. Carson-Walter, E. B., Watkins, D. N., Nanda, A., Vogelstein, B., Kinzler, K. W. & St Croix, B. (2001) *Cancer Res.* **61,** 6649–6655.
12. Liu, S. & Leppla, S. H. (2003) *J. Biol. Chem.* **278,** 5227–5234.
13. Whittaker, C. A. & Hynes, R. O. (2002) *Mol. Biol. Cell* **13,** 3369–3387.
14. Shimaoka, M., Takagi, J. & Springer, T. A. (2002) *Annu. Rev. Biophys. Biomol. Struct.* **31,** 485–516.
15. Bell, S. E., Mavila, A., Salazar, R., Bayless, K. J., Kanagala, S., Maxwell, S. A. & Davis, G. E. (2001) *J. Cell Sci.* **114,** 2755–2773.
16. Milne, J. C., Blanke, S. R., Hanna, P. C. & Collier, R. J. (1995) *Mol. Microbiol.* **15,** 661–666.
17. Klostermann, A., Lutz, B., Gertler, F. & Behl, C. (2000) *J. Biol. Chem.* **275,** 39647–39653.