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Anthrax Toxin Protective Antigen Variants That Selectively Utilize either the CMG2 or TEM8 Receptors for Cellular Uptake and Tumor Targeting*[S]

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Kuang-Hua Chen[‡], Shihui Liu[‡§], Clinton E. Leysath[‡1], Sharmina Miller-Randolph[‡], Yi Zhang[‡], Rasem Fattah[‡], Thomas H. Bugge[§], and Stephen H. Leppla[‡2]

From the [‡]Laboratory of Parasitic Diseases, NIAID, National Institutes of Health, Bethesda, Maryland 20892-3202 and the [§]Proteases and Tissue Remodeling Section, Oral and Pharyngeal Cancer Branch, NIDCR, National Institutes of Health, Bethesda, Maryland 20892

The protective antigen (PA) moiety of anthrax toxin binds to cellular receptors and mediates the translocation of the two enzymatic moieties of the toxin to the cytosol. Two PA receptors are known, with capillary morphogenesis protein 2 (CMG2) being the more important for pathogenesis and tumor endothelial marker 8 (TEM8) playing a minor role. The C-terminal PA domain 4 (PAD4) has extensive interactions with the receptors and is required for binding. Our previous study identified PAD4 variants having enhanced TEM8 binding specificity. To obtain PA variants that selectively bind to CMG2, here we performed phage display selections using magnetic beads having bound CMG2. We found that PA residue isoleucine 656 plays a critical role in PA binding to TEM8 but has a much lesser effect on PA binding to CMG2. We further characterized the role of residue 656 in distinguishing PA binding to CMG2 *versus* TEM8 by substituting it with the other 19 amino acids. Of the resulting variants, PA I656Q and PA I656V had significantly reduced activity on TEM8-expressing CHO cells but maintained their activity on CMG2-expressing CHO cells. The preference of these PA mutants for CMG2 over TEM8 was further demonstrated using mouse embryonic fibroblast cells and mice deficient in the CMG2 and/or the TEM8 receptors. The structural basis of the alterations in the receptor binding activities of these mutants is also discussed.

Anthrax toxin is composed of three nontoxic proteins: protective antigen (PA),[3] lethal factor (LF), and edema factor (EF), which are released from *Bacillus anthracis* as monomers and assemble into toxic complexes on the surface of animal cells (1–3). PA is the moiety which binds to cell surface receptors and facilitates entry of the enzymatic EF and LF moieties into the host cell cytosol. Two anthrax toxin receptors have been identified; capillary morphogenesis protein 2 (CMG2, or anthrax toxin receptor 2) is the physiologically relevant toxin receptor mediating most of the *in vivo* toxicity of the toxin, whereas tumor endothelial marker 8 (TEM8, or anthrax toxin receptor 1) functions only as a minor anthrax toxin receptor (4–8). Upon binding to the toxin receptors, PA is cleaved at the sequence ^{164}RKKR^{167} by cell surface furin or furin-like proteases (9, 10), yielding the C-terminal 63-kDa fragment (PA63), which assembles to form a ring-shaped oligomer. The PA63 oligomer then binds LF and/or EF, and the toxin complex is internalized by receptor-mediated endocytosis. LF and EF are translocated into the cytosol from early or late endosomes to exert their cytotoxic effects. Therefore, PA plays the crucial role in anthrax toxin pathogenesis, serving as the delivery vehicle for translocation of LF and EF into the cytosol of the cell.

The absolute requirement for cell surface proteolytic activation of PA for the toxin's action prompted our group and others to reengineer PA to make it depend on tumor-associated proteases for activation, thereby achieving high specificity for tumors (11–15). In a recent example, we demonstrated that a mixture of two PA variants, one requiring activation by matrix metalloproteases and the other by urokinase plasminogen activator, caused strong anti-tumor responses when administered with LF (13, 16). Furthermore, when this "IC2-PA" mixture was administered with drugs that block induction of neutralizing antibodies, the toxin suppressed tumor growth for many weeks (16). Specificity for tumors might also be achieved by altering the receptor binding specificity of PA (17, 18). Thus, we previously obtained PA mutants that preferentially bind to TEM8 rather than CMG2 (17). This was done using a T7 phage library displaying randomly mutated variants of PA domain 4 (PAD4), the C-terminal domain containing amino acids 596–735 that is largely responsible for receptor binding (19). In this study, we used CMG2 bound to magnetic beads to select PAD4 mutants that bind specifically to the CMG2 receptor. We show here that

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[1] Present address: Center for Innovation in Advanced Development and Manufacturing, Office of Public Health Preparedness and Response, Texas A&M Health Science Center, 8441 State Hwy. 47, Suite 3200, Bryan, TX 77807.

[2] To whom correspondence should be addressed: Laboratory of Parasitic Diseases, Bldg. 33, Rm. 1W20B, NIAID, National Institutes of Health, Bethesda, MD 20892-3202. Tel.: 301-594-2865; Fax: 301-480-0326; E-mail: sleppla@niaid.nih.gov.

[3] The abbreviations used are: PA, protective antigen; LF, lethal factor; EF, edema factor; ECD, extracellular domain; 8-oxo-dGTP, 8-oxo-2′-deoxyguanosine triphosphate; dPTP, 6-(2-deoxy-β-D-ribofuranosyl)-3,4-dihydro-8H-pyrimido-[4,5-c][1,2]oxazin-7-one triphosphate; MEF, mouse embryonic fibroblast; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

TABLE 1

Specific binding of bacteriophage displaying randomly mutated PAD4 variants to CMG2 and TEM8 extracellular domains

The binding of bacteriophage to the CMG2 and TEM8 extracellular domains bound to magnetic beads (Dynabeads) is detailed under "Experimental Procedures." After binding of bacteriophage, the mixture was washed five times with binding and washing buffer for a total of 10 min, and bacteriophage were eluted with elution buffer and titered. Expt., experiment.

Treatments	Phage bound to Dynabeads
Expt. 1: CMG2 magnetic beads	
Control[a]	7.5×10^2
CMG2-magnetic beads	6.6×10^4
CMG2-magnetic beads + PA[b]	5.6×10^3
Expt. 2: TEM8 magnetic beads	
Control[a]	7.4×10^2
TEM8-magnetic beads	7.0×10^4
TEM8-magnetic beads + PA[b]	5.6×10^3

[a] Binding of bacteriophage to Dynabeads without the addition of an extracellular domain.

[b] 100 ng of PA was mixed with bacteriophage lysate before adding to the Dynabeads.

TABLE 2

Binding of PAD4 T7Select bacteriophage to CMG2 and TEM8 extracellular domains in successive rounds of panning

Experimental procedures were the same as described in Table 1, except the magnetic beads were washed for 10 min, 2 h, 5 h, and 20 h in the first, second, third, and fourth rounds of panning, respectively. Expt., experiment.

Round of panning	Phage bound to Dynabeads
Expt. 1: CMG2-magnetic beads	
1st	3.8×10^4
2nd	3.8×10^5
3rd	2.3×10^7
4th	8.1×10^6
Expt. 2: TEM8-magnetic beads	
1st	7.0×10^4
2nd	1.2×10^7
3rd	1.1×10^7
4th	4.5×10^7

residue Ile-656 is required for strong binding to TEM8 and that substitution of Ile-656 with certain amino acids, such as Gln, produces PA variants that have high affinity for CMG2 but little to no affinity for TEM8.

Results

Selection of PAD4 Mutants That Bind to CMG2—Our previous study (17) identified PAD4 mutants that bind differentially to TEM8 and CMG2. This was accomplished by bacteriophage display of a library of randomly mutated PAD4 variants. PA variants were selected using plates coated with mouse monoclonal antibody 14B7, which mimics the receptors by binding to the same PAD4 surface. Here, we developed an improved panning procedure using soluble forms of the extracellular domains of CMG2 and TEM8 bound to magnetic beads (Dynabeads TALON). The specific binding of the phage-displayed PAD4 to each type of beads was verified by showing that mixing the phage with 100 ng of PA before adding the mixture to the beads reduced phage binding by 90% (Table 1). To enrich for PAD4 variants having high affinity, we increased the stringency of washing in each successive amplification round, using washing times of 10 min, 2 h, 5 h, and 20 h. The number of phage increased 600-fold by the third round of panning, suggesting that the initial phage library was heavily mutagenized. Interestingly, the fourth round of panning on CMG2 resulted in a 65% decrease in phage numbers (Table 2), possibly because the highest possible affinity had already been reached by the third round, and the prolonged 20-h washing removed some of these tightly binding phage. After the fourth round of panning, individual bacteriophage plaques were picked and sequenced.

A total of 104 phage clones were sequenced over the 420-bp region corresponding to PA amino acids 596–735. These DNA sequences were aligned in a spreadsheet, which is provided as supplemental File 1. The resulting amino acid sequences of the clones are shown in supplemental File 2. Among the resulting total of 43,680 bp examined, a total of 616 changes were found, for a mutation rate of 1.4%. However, this value and the subsequent analyses are for the phage library after selection for binding to CMG2. This selection will eliminate both the large fraction of sequences coding for misfolded or unstable proteins and those with specific surface residue changes that decrease receptor recognition. The selection is also expected to enrich for silent mutations. In fact, we found that the number of substitutions in codon positions 1–3 was 207, 165, and 244, respectively. Thus, substitutions in the third position, which are less likely to change the amino acid, were enriched, consistent with the expected elimination of clones having the most common mutagenic events, amino acid changes that decrease structure and/or function.

Although mutation frequencies and other numerical values were calculated, these can be viewed only as estimates. Comparison of the DNA and amino acid sequences shows that many clones are related. The bp changes seen in multiple clones must have occurred in an early PCR cycle and then expanded into many progeny sequences. This illustrates that the clones are not independent, as would be needed to achieve an accurate statistical analysis.

Because dPTP causes A to G and T to C transitions and 8-oxo-GTP causes A to C and T to G transversions (20), a broad spectrum of amino acid substitutions should be generated, although codons containing G and C will be less affected. We found instead that nearly all of the changes were those expected for dPTP: 379 A to G and 135 T to C. Fig. 1 compares the frequencies we obtained with those reported in the original description of these two mutagenic nucleotides (20). It should be noted that those authors used high concentrations of dPTP and 8-oxo-GTP (500 μM each *versus* the 10 and 50 μM, respectively, which we used), and even when they combined the two, the effects of the 8-oxo-GTP were quite limited, so that A to G and T to C transversions greatly predominated, similar to our results.

Although there were multiple amino acid mutations in nearly every clone from the CMG2-selected library (supplemental File 2), strikingly, we found that mutation of Ile-656 to Val, in the region between 4β6 and 4β7 (amino acids 656–665), occurred in nearly all of the clones, indicating that residue Ile-656 is critical for binding of PA to the CMG2 receptor. In contrast, the TEM8-selected library showed a greater diversity of substitutions (supplemental File 2). However, residue Glu-654, in the same region as Ile-656, was frequently mutated. Thus, of the 95 clones sequenced, 27 have Glu-654 substituted by either Gly or Ala, indicating that this residue may be critical for binding of PA to TEM8.

PAD4 Variants Having Substitutions at Ile-656—The role of Ile-656 in binding of PA to TEM8 was further explored by

A.

From ↓	To G	A	T	C
G	-	5.0	0.8	7.3
A	61.5	-	1.0	0.2
T	0	0.5	-	21.9
C	0.2	0	1.6	-

B.

From ↓	To G	A	T	C
G	-	7.8	0	0
A	40.7	-	0.2	6.6
T	1.8	0	-	37.6
C	0.7	0	5.4	-

FIGURE 1. **Nucleotide changes induced by mutagenic PCR.** Values are the percentages of the total nucleotide substitutions of each type. *A*, results of this study for bacteriophage selected for binding to CMG2 ECD. Values were calculated from the spreadsheet provided as supplemental File 1. *B*, data adapted from the original study using dPTP and 8-oxo-dGTP (20), provided for comparison.

TABLE 3

Toxicity of PA Ile-656 variants to CHO cells

PA variants containing all 20 amino acids at position 656 were tested for toxicity to CHO cells. Values in the table are EC_{50}, the concentrations of the PA proteins that killed 50% of cells when combined with 100 ng/ml FP59. The analyses were performed three times, and the averages are listed.

PA variant	EC_{50} on CHO cells[a] WTP4	TEM8-T4	CMG2-C4	Selectivity for CMG2 *versus* TEM8[b]
		ng/ml		
PA (native)	0.14*	0.21	0.05	4
PA I656Q	0.48*	>83.3	0.05	>1667
PA I656S	0.42	>83.3	0.07	>1190
PA I656N	1.10	>83.3	0.12	>694
PA I656H	4.11	>83.3	0.17	>490
PA I656E	1.71	>83.3	0.17	>490
PA I656A	3.87	>83.3	0.26	>320
PA I656G	1.83	>83.3	0.29	>287
PA I656K	9.27	>83.3	0.31	>269
PA I656C	0.63*	17.33	0.10	173
PA I656M	0.24	4.70	0.05	99
PA I656Y	2.13	13.61	0.14	99
PA I656L	0.22	3.71	0.04	92
PA I656F	1.76	7.29	0.12	61
PA I656T	0.28	1.59	0.03	53
PA I656D	15.33	>83.3	1.91	>44
PA I656P	2.76	5.25	0.22	24
PA I656V	0.35	1.34	0.06	22
PA I656R	>83.3	>83.3	5.33	>16
PA I656W	>83.3	>83.3	>83.3	NC[c]

[a] In EC_{50} values, S.D. values are indicated as follows. No asterisk indicates that S.D. = <0.5 × the absolute value or cannot be calculated, and one asterisk indicates that S.D. = 0.5–1.0 × the absolute value.
[b] Ratio: (EC_{50} for TEM8-T4)/(EC_{50} for CMG2-C4). Variants are sorted according to this value.
[c] Not able to be calculated.

mutating Ile-656 to all of the other 19 amino acids. Substitutions of Ile-656 with Leu, Met, Gln, Ser, Thr, Val, and Tyr were well tolerated, because these proteins retained cytotoxicity to the wild type CHO WTP4 cells (Table 3). Intriguingly, whereas these Ile-656 mutants also retained activity to CHO CMG2-C4 cells, the CHO cell line that overexpresses only CMG2, all of these PA variants displayed greatly decreased cytotoxicity to CHO TEM8-T4 cells, which overexpress only TEM8. In particular, PA I656Q and I656S showed no cytotoxicity to CHO TEM8-T4 cells at the highest concentration tested, 80 ng/ml. Therefore, PA I656Q and I656S are highly selective for CMG2. All other variants showed decreased cytotoxicity to all three types of CHO cells. PA I656W showed no cytotoxicity to the cell lines even at the highest concentration (80 ng/ml) tested (Table 3). Nearly all of the Ile-656 substitution variants showed a much greater loss of cytotoxicity toward the CHO TEM8-T4 cells than toward CHO CMG2-C4 and CHO WTP4 cells. A rationale for preferential binding by these variants is provided under "Discussion."

TABLE 4

Toxicity of PA E654 variants to CHO cells

PA variants containing all 20 amino acids at position 654 were tested for toxicity to CHO cells. Values in the table are EC_{50}, the concentrations of the PA proteins that killed 50% of cells when combined with 100 ng/ml FP59. The analyses were performed three times, and the averages are listed.

PA variant	EC_{50} on CHO cells[a] TEM8-T4	CMG2-C4	Selectivity for TEM8 *versus* CMG2[b]
	ng/ml		
PA (native)	0.52	0.39*	0.7
PA E654C	0.16	0.81*	4.9
PA E654N	0.16	0.68	4.3
PA E654Q	0.13	0.39	2.9
PA E654S	0.10	0.26*	2.6
PA E654H	0.22	0.53	2.4
PA E654F	0.63	1.43	2.3
PA E654Y	0.16	0.36	2.3
PA E654W	0.15*	0.34	2.2
PA E654T	0.42	0.89	2.1
PA E654I	0.73	1.27	1.7
PA E654M	0.35	0.58	1.7
PA E654A	0.54	0.88	1.6
PA E654L	0.52	0.70	1.3
PA E654G	0.58	0.59	1.0
PA E654V	0.19*	0.20	1.0
PA E654R	2.40	1.12	0.5
PA E654P	7.20	2.80	0.4
PA E654D	2.40	0.83	0.3
PA E654K	2.50	0.50	0.2

[a] In EC_{50} values, S.D. values are indicated as follows. No asterisk indicates that S.D. = <0.5 × the absolute value or cannot be calculated, and one asterisk indicates that S.D. = 0.5–1.0 × the absolute value.
[b] Ratio: (EC_{50} for CMG2-C4)/(EC_{50} for TEM8-T4). Variants are sorted according to this value.

PAD4 Variants Having Substitutions at Glu-654—To further characterize the role of position 654 in binding to CMG2 (as indicated in supplemental File 2), we mutated Glu-654 to all of the other 19 amino acids. In many cases, the substitutions led to a greater reduction in potency toward CHO CMG2-C4 than toward CHO TEM8-T4 cells (Table 4), although the losses in potency were smaller than those caused by substitutions at Ile-656 (Table 3). Only the substitution of Val was fully tolerated, as shown by the retention of toxicity toward both cell types. In contrast, substitution of Ala, Cys, Gly, His, Asn, Gln, Ser, Thr, Trp, and Tyr caused decreases in potency toward CHO CMG2-C4 cells that were not paralleled by decreases in potency toward CHO TEM8-T4. In fact, many of these substitutions appeared to cause a slight increase in potency toward the CHO TEM8-T4 cells, as seen particularly with the E654Q, E654V, and E654W variants. These variants have a 2–3-fold higher toxicity toward CHO TEM8-T4 than does native PA,

TABLE 5

Toxicity of PA variants combining substitutions at several sites

PA variants having R659S and M662R substitutions along with substitution of each of the other 19 amino acids at position 654 were tested for toxicity to CHO cells. The three-letter designation identifies the non-native substitutions at positions 654, 659, and 662. Values in the table are EC_{50}, the concentration of the PA protein that produces 50% killing when combined with 100 ng/ml FP59. The analyses were performed three times, and the averages are listed.

PA variant		EC_{50} on CHO cells[a]		Selectivity for TEM8 *versus* CMG2[b]
Designation	Substitution (in addition to R659S/M662R)	TEM8-T4	CMG2-C4	
		ng/ml		
PA (native)		0.40	0.16	0.4
MSR	E654M	0.60	6.83	11.4
TSR	E654T	0.35	3.00	8.5
QSR	E654Q	0.44	3.20	7.3
GSR	E654G	0.49	3.47	7.1
YSR	E654Y	0.63	4.33	6.8
VSR	E654V	0.71	3.93	5.5
RSR	E654R	15.67	>83	>5.3
LSR	E654L	3.53	17.3	4.9
FSR	E654F	0.62	2.97	4.8
ISR	E654I	2.00	9.33	4.7
NSR	E654N	0.43	1.73	4.1
CSR	E654C	6.40	17.0	2.7
SSR	E654S	0.35	0.91*	2.6
KSR	E654K	40.2	>83	>2
HSR	E654H	0.21	0.38	1.8
ASR	E654A	0.36	0.60	1.7
WSR	E654W	0.47	0.63	1.3
DSR	E654D	8.00	5.00	0.6
PSR	E654P	>83	>83	NC[c]

[a] In EC_{50} values, S.D. values are indicated as follows. No asterisk indicates that S.D. = <0.5 × the absolute value or cannot be calculated, and one asterisk indicates that S.D. = 0.5–1.0 × the absolute value.

[b] Ratio: (EC_{50} for CMG2-C4)/(EC_{50} for TEM8-T4). Variants are sorted according to this value.

[c] Not able to be calculated.

TABLE 6

Cytotoxicity of PA variants to CMG2- and TEM8-null MEFs

PA variants were tested for cytotoxicity to the indicated MEF cells. Values in the table are EC_{50}, the concentrations of the PA proteins that killed 50% of cells when combined with 400 ng/ml FP59. The analyses were performed two additional times with results similar to those presented. Expt., experiment.

	EC_{50} on MEFs			
PA variant	WT	$CMG2^{-/-}$	$TEM8^{-/-}$	$CMG2^{-/-}/TEM8^{-/-}$
		ng/ml		
Expt. 1				
PA (native)	0.8	4.8	0.9	>1000
PA I656Q	0.9	>1000	0.9	>1000
PA I656V	1.0	7.0	1.1	>1000
PA I656W	>1000	>1000	>1000	>1000
Expt. 2				
PA (native)	0.9	17	1.0	
PA MSR	9.5	19	>1000	
PA QSR	9.0	16	700	
PA TSR	3.5	7	130	

indicating that the Glu-654 residue plays an important role in determining the relative affinities for the two receptors.

PAD4 Variants Having Substitutions in Multiple Residues—Our previous study identified the PA R659S/M662R variant (also named PA D7) as having selectivity for TEM8 *versus* CMG2 (17). To examine the effects of combining substitutions, we selected PA variants combining all 19 amino acids replacing Glu-654 with the R659S/M662R substitutions. All 19 substitution mutants showed decreased toxicity toward CHO CMG2-C4 cells (Table 5). Particularly, the PA variants KSR, PSR, and RSR were nontoxic to CHO CMG2-C4 cells when added at 80 ng/ml, the highest concentration tested. In contrast, PA variants ASR, GSR, HSR, MSR, NSR, QSR, SSR, TSR, and WSR had potencies equal to or slightly greater than that of native PA toward CHO TEM8-T4 cells (Table 5). For example, PA TSR was only slightly more toxic to CHO TEM8-T4 cells than native PA but 18-fold less toxic to CHO CMG2-C4 cells. The PA QSR, MSR, and GSR variants were similar to PA TSR, being only slightly less selective. Based on these data, PA MSR, QSR, and TSR were selected for further study.

Cytotoxicity of PA Variants to Receptor-deficient Mouse Embryonic Fibroblasts (MEFs)—Although the CHO cell lines expressing a single receptor were essential in determining the preference of PA variants for CMG2 and TEM8, we wished to verify the behavior of the PA proteins on other cell types. Mice having each receptor deleted and cells isolated from them provide a well defined genetic system for examining the receptor specificity of the PA variants (7, 8, 21). Cytotoxicity tests of the PA variants having substitutions at Ile-656 (from Table 3) on MEFs isolated from these mice clearly demonstrated the high specificity of the PA Ile-656 variants for CMG2 (experiment 1 in Table 6). The WT MEF cells appear to express both receptors, whereas the $CMG2^{-/-}$ MEFs are highly resistant to PA I656Q while remaining sensitive to native PA and other variants that can use the remaining TEM8 receptor. Notably, the MEF cells from mice deleted for both receptors are highly resistant to all of the PA variants, consistent with our prior evidence that there are no physiologically significant receptors other than CMG2 and TEM8 (8, 21).

In a parallel experiment (Table 6, experiment 2) we again used the MEF and showed that the PA triple substitution variants, such as PA TSR, exert their toxicity through the TEM8 receptor, because absence of the CMG2 receptor has little effect on their potency, whereas absence of the TEM8 receptor decreases potency >100-fold. Taken together, these data using MEF cells confirm the selectivity of the PA variants that was seen in Tables 3 and 5.

Affinity of PA Variants for Receptors as Determined by Schild Plot Analyses—It was expected that the differences in toxicity of the PA variants as described above result principally from differences in receptor binding affinities. To examine this hypothesis, apparent affinities were measured by competitive Schild plot analyses. These analyses require that the protein ligand be a nontoxic variant of the protein of interest. Nontoxic variants of the PA I656Q, PA I656V, and PA TSR proteins were constructed by replacing their furin cleavage sites, ^{164}RKKR^{167}, with the uncleavable sequence PGG of the previously characterized PA-U7 protein (12). The resulting PA-U7 I656Q, PA-U7 I656V, and PA-U7 TSR were used as nontoxic receptor binding competitors. Reciprocal plots of the midpoints of the dose-response curves performed in varying amounts of the competitors yielded apparent dissociation constants (K_d) for the apparent affinity of the competitors to the PA receptors. These analyses showed that PA I656Q retained affinity for the CMG2 receptor but greatly lost affinity for the TEM8 receptor (Table 7, experiment 1). Although PA I656V retained affinity for CMG2, it had moderately decreased affinity for TEM8. Conversely, PA-U7 TSR showed a decrease in affinity to CMG2 (Table 7, experiment 2), consistent with its preference for TEM8.

Toxicity of PA Variants to CMG2 and TEM8 Knock-out Mice—To explore whether the differing receptor specificities of the PA variants described above applied *in vivo*, we challenged CMG2$^{-/-}$ and/or TEM8$^{-/-}$ mice and their littermate control mice using 20 μg of PA variants plus 20 μg of FP59 (fusion protein of LF and *Pseudomonas* exotoxin A) (Fig. 2). All of the WT and TEM8$^{-/-}$ mice were killed by native PA + FP59 within 24 h (Fig. 2*A*), whereas most of the CMG2$^{-/-}$ mice survived to 48 h but succumbed to the second dose given at 48 h within the following 24 h, consistent with CMG2 being the major toxin receptor mediating mouse lethality (8, 21). Interestingly, all of the CMG2$^{-/-}$ mice survived two doses of the CMG2-selective PA I656Q + FP59 (Fig. 2*B*), whereas all of their littermate control mice and TEM8$^{-/-}$ mice died, mostly within 24 h after the toxin challenge, demonstrating that the high specificity of PA I656Q for CMG2 extends to the case of intact mice. The moderate increase in specificity of PA I656V to CMG2 was also confirmed, because 50% the CMG2$^{-/-}$ mice survived to 75 h after receiving two doses of PA I656V + FP59 (Fig. 2*C*), whereas all CMG2$^{-/-}$ mice had succumbed by this time after challenge with the native PA + FP59 (Fig. 2, compare *A* and *C*).

Comparison of the potency of the putatively TEM8-specific PA TSR variant toward wild type and CMG2-null mice produced interesting results (Fig. 2*D*). The PA TSR variant was much less toxic toward wild type mice than native PA, consistent with the CMG2 receptor being the main determinant of toxin sensitivity. However, PA TSR was more potent toward the CMG2-null mice than toward wild type mice. In the CMG2-null mice, death must result from targeting the TEM8 receptor in certain (unidentified) tissues, and PA TSR appears to do this more efficiently than native PA (also see "Discussion").

Tumoricidal Activity of PA Variants to Human HeLa Xenografts in Mice—To examine the effects of altering the receptor specificity of PA on its tumor-targeting ability, two of the PA variants were tested against HeLa cell xenografts in mice, in comparison with the previously described PA-L1 variant. The PA variants used here contained the metalloprotease-specific L1 sequence at the furin site so as to achieve tumor specificity, and the effector used was LF rather than FP59. Mice bearing solid intradermal tumor nodules constituting ~0.05% of the total body mass were challenged by intraperitoneal injection of six doses of the toxins at 2- or 3-day intervals. At the doses used, mouse body weights did not differ between the groups (Fig. 3*A*). In addition, the mice showed no outward sign of illness or gross abnormalities. However, large differences were seen in the effect of the PA variants on tumor size. Both PA-L1 and PA-L1 I656Q had strong and similar anti-tumor activities, reducing tumor size by >80% compared with PBS-treated tumors (Fig.

TABLE 7

Apparent affinities of PA variants for CMG2 and TEM8

Analyses were performed as described under "Experimental Procedures." PA-U7, PA-U7 I656V, PA-U7 I656Q, and PA-U7 TSR were used as competitors for PA in cytotoxicity assays with the two cell lines listed. Schild plot analyses determined the apparent affinities of the competitors for the receptors on the two cell lines. Expt., experiment.

Competitor	K_d on cell lines	
	CHO CMG2-C4	CHO TEM8-T4
	nM	
Expt. 1		
PA-U7	8	25
PA-U7 I656V	8	46
PA-U7 I656Q	8	>240
Expt. 2		
PA-U7	12	35
PA-U7 TSR	48	30

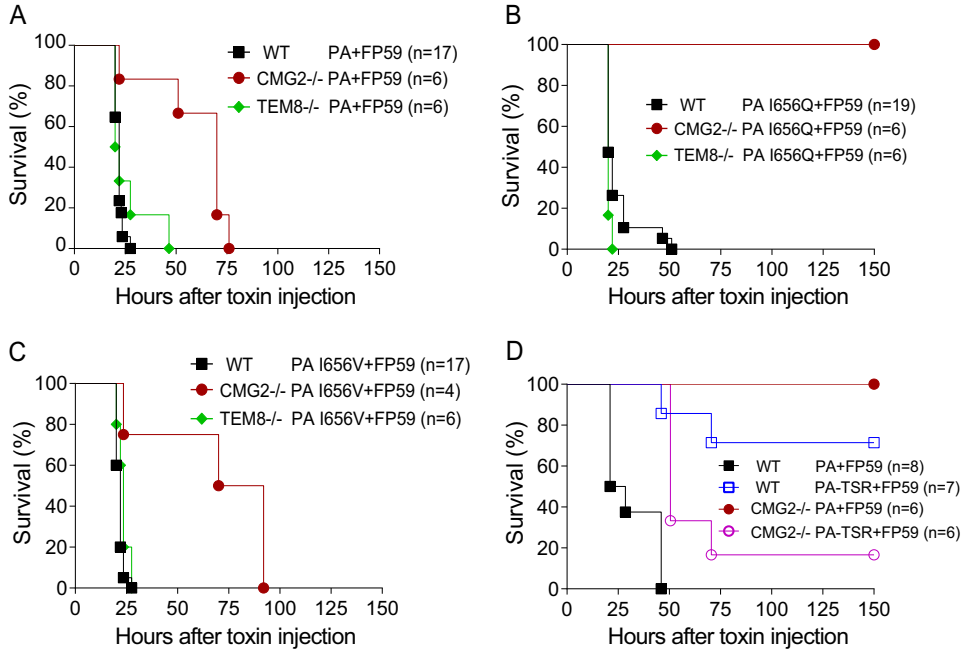

FIGURE 2. **Lethality of PA variants to CMG2$^{-/-}$ and/or TEM8$^{-/-}$ mice.** CMG2$^{-/-}$ and TEM8$^{-/-}$ mice and their littermate control mice were challenged intraperitoneally with two doses of 20 μg of PA or PA variants along with 20 μg of FP59 (in 0.5 ml of PBS) with a 2-day interval. Signs of malaise were observed twice daily for 1 week following injections. *A–D* show results for mice challenged with native PA, PA I656Q, PA I656V, and PA TSR, respectively. A log-rank (Mantel-Cox) test was used for survival curve comparison. *A*, WT *versus* CMG2$^{-/-}$, $p = 0.0003$; WT *versus* TEM8, $p = 0.923$. *B*, WT *versus* CMG2$^{-/-}$, $p < 0.0001$; WT *versus* TEM8, $p = 0.107$. *C*, WT *versus* CMG2$^{-/-}$, $p = 0.0009$; WT *versus* TEM8, $p = 0.135$. *D*, WT PA-TSR + FP59 *versus* CMG2$^{-/-}$ PA-TSR + FP59, $p = 0.0409$.

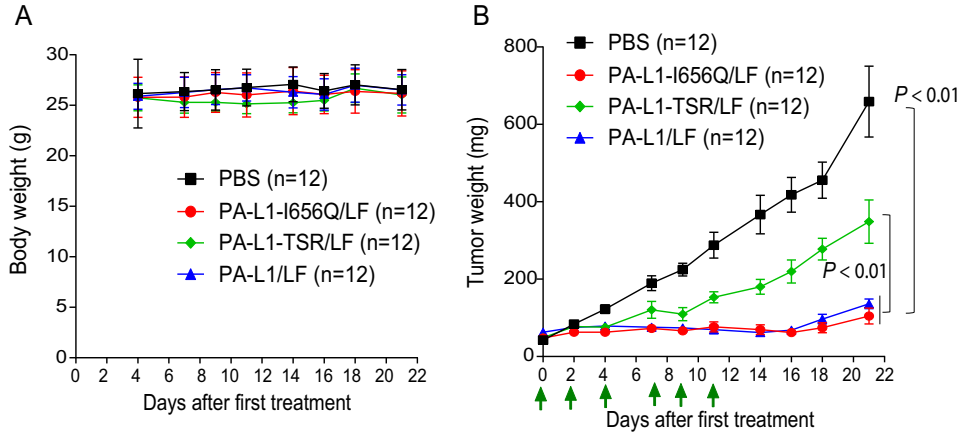

FIGURE 3. **Tumoricidal activity of PA variants to HeLa xenograft in mice.** Mice bearing HeLa xenografts were injected intraperitoneally with PBS (♦), 15 μg of PA-L1 + 5 μg of LF (■), 15 μg of PA-L1 I656Q + 5 μg of LF (▲), or 30 μg of PA-L1 TSR + 10 μg LF (●). Both body and tumor weights were measured every 2 days after the first injection of toxins. *A*, body weight changes during treatment period. *B*, weights of intradermal tumor nodules are expressed as mean of tumor weight. Signs of malaise were observed twice daily for 3 weeks following injections. Group sizes are indicated for each toxin. Student's *t* test was used to calculate differences between groups.

3*B*). In contrast, the tumors showed less response to treatment with PA-L1 TSR, with only a 50% reduction of tumor size occurring (Fig. 3*B*). These data are consistent with other evidence that targeting of tumors is most effective when PA acts through CMG2 rather than TEM8.

Discussion

This study extends our prior work to identify variants of the anthrax toxin PA that discriminate between TEM8 and CMG2, the two related proteins that act as cell surface PA receptors (17). TEM8 was the first toxin receptor to be described (4) and continues to receive attention as a potential tumor marker. CMG2 appears to be expressed more widely in tissues, has a higher affinity for PA (6), and in our work has been found to be more important for both bacterial pathogenesis (8, 22) and tumor targeting (16, 21).

Our prior study used phage display of a diverse library of domain 4 variants to isolate sequences with preference for TEM8. A competitive selection procedure was used to discriminate against sequences that bind to CMG2. In practice, a large fraction of the resulting clones showed decreased binding to CMG2 and a larger relative loss in binding to TEM8. This was probably unavoidable because of the greater affinity of PA for CMG2 than for TEM8. Fortunately, the study did identify a rare clone, PA D7, having the desired property of retaining full toxicity toward TEM8-expressing cells while having ~20-fold lower potency toward CMG2-expressing cells. However, this PA D7 was 10-fold less potent than native PA on nearly all types of tumor cells, suggesting that CMG2 is the more common and relevant receptor and that PA D7 would be of limited value as a tumor-targeting agent.

In the current study, we used an improved protocol to separately select phage-displayed sequences showing strong binding to either TEM8 or CMG2. Given increasing evidence that CMG2 is the more relevant receptor for tumor-targeting approaches and our efforts to re-engineer anthrax toxin for this purpose (16), we focused on analysis of the CMG2-preferring clones. Strikingly, almost all of the independent mutants (85 of 86) contained the I656V substitution, pointing to this residue as a key element controlling binding to CMG2. The mutation causing the Ile-656 codon, ATA, to become GTA, encoding Val, was one of the abundant A to G transitions, presumably induced by dPTP. As detailed below, it was subsequently found (Table 3) that many substitutions at Ile-656 other than Val cause a stronger selectively for CMG2, but few of these could be generated by single A to G or T to C bp changes of the type generated by the mutagenic process. Apparently, even the 22-fold selectivity of the I656V substitution was sufficient to lead to enrichment during the panning steps.

The role of the PA Ile-656 residue was rigorously examined by substitution with all other 19 amino acids, with results confirming the key role of this residue in discriminating between TEM8 and CMG2. Thus, nearly all Ile-656 substitutions retained high potency on CMG2-expressing cells while showing large losses in activity toward TEM8. A number of these PA variants had increases in CMG2 selectivity of >100-fold on cells. The PA I656Q variant had a selectivity increase of >1000 and was therefore selected for further analysis. Competitive Schild plot measurements showed that PA I656Q had a >10-fold decrease in apparent affinity for TEM8 and no change in apparent affinity for CMG2. The smaller change seen in affinity measurements than in cell culture potency measurements may reflect the fact that in the affinity measurements, there is a 1:1 PA/receptor interaction, whereas in the cell culture assay, PA must bind to multiple receptors to create the oligomeric channel that binds LF (or other effectors).

The PA Ile-656 residue appears to be partially buried within domain 4, lying behind the small loop (amino acids 679–693) between 4β8 and 4β9 (23). This small loop directly contacts the receptors, as revealed in the structure of the CMG2-PA complex (Protein Data Bank code 1TZN). (See also Fig. 2 of our previous report (17).) Within this loop lies the key Asp-683 residue that chelates the Mg^{2+} ion of CMG2 and TEM8. The PA Ile-656 residue is partially exposed on the surface and is close to the Val-115 residue of CMG2, which in TEM8 is replaced by a Gly. The strong effect of PA Ile-656 in discriminating between CMG2 and TEM8 may reflect the different

interactions of the PA 656 and CMG2/TEM8 115 residues. Thus, binding of PA to TEM8 appears to be optimal for PA variants having an amino acid that branches at the β-carbon of PA residue 656, because this maintains maximum contact between the two interfacing polypeptide chains. Indeed, Val and Thr variants at position 656 retained the greatest activity for TEM8 of all of the mutants tested (Table 3). Loss of this branching greatly reduces TEM8 binding while having less of an effect on CMG2. CMG2, which has Val at residue 115, appears to be much more forgiving in binding to PA having various residues at position 656. It should also be considered that substitutions at position 656 could exert their strong effects at least in part by altering the overall conformation of domain 4. This explanation would be consistent with the large losses in potency seen in most of the PA variants having substitutions at Asn-657 (17), the residue adjacent to Ile-656 and one that is also mostly buried within the protein.

The current study also sought again to identify PA variants with selectivity for TEM8. As explained earlier, this was not expected to be easily achieved because PA affinity for TEM8 is about 10-fold lower than for CMG2. The best variant obtained, PA TSR, retained normal activity toward TEM8 while losing activity toward CMG2, with the result being an increase in TEM8 selectivity of about 50-fold. It was interesting that PA TSR was more toxic to CMG2-deficient mice than to intact mice, suggesting that in the normal mice, toxin is diverted to the more abundant CMG2-containing tissues (although it has reduced affinity for these), thus sparing the unidentified TEM8-expressing tissues through which the toxin kills the mice.

A key result of this work was identification of the I656Q substitution. In the tumor-treatment experiment presented here, PA-L1 I656Q was as potent as PA-L1, consistent with its retention of binding to CMG2. As seen in the mouse toxicity tests, the I656Q variant was much less toxic to mice lacking the CMG2 receptor (and having only the TEM8 receptor) than to wild type mice, consistent with its loss of affinity for TEM8. This decreased toxicity toward TEM8 suggests that this CMG2-specific variant will have an improved therapeutic index compared with PA when used as a component of tumor-targeting agents. In ongoing work, the I656Q substitution is being placed into the previously described IC2-PA and "octamer" drugs (13, 16, 24), with an expectation of improving their specificity and, therefore, their therapeutic indices.

In summary, we have demonstrated that PA residue 656 is critical in determining PA binding to the CMG2 and TEM8 receptors and that PA I656Q is a novel CMG2-specific PA variant. PA I656Q should be a useful tool in the further development of CMG2 receptor-specific tumor-targeted anthrax toxins.

Experimental Procedures

Reagents—Enzymes for DNA manipulations and modifications were purchased from New England Biolabs (Beverly, MA). Dynabeads TALON was obtained from Invitrogen. PA and FP59 were prepared in our laboratory as described (25–27). The extracellular domain (ECD) of CMG2 (amino acids 40–218) was produced in *Escherichia coli* with an N-terminal His_6 tag (17, 23). The ECD of TEM8 (amino acids 35–227) with a C-terminal His_6 sequence was expressed in CHO cells. The latter protein was concentrated and purified from the culture medium with a nickel-nitrilotriacetic acid column (Qiagen, Valencia, CA) and further purified by MonoQ column chromatography (GE Healthcare). Minimum essential medium α, HEPES buffer, hygromycin B, Hanks' balanced salt solution, and FBS were from Invitrogen. The mutagenic nucleoside triphosphates 8-oxo-dGTP and dPTP were purchased from Trilink Biotechnologies (San Diego, CA). Other chemical reagents were obtained from Sigma.

Construction of a Randomly Mutated PAD4 Phage Display Library—The DNA sequence encoding PAD4 was obtained by PCR from plasmid pYS5 using forward and reverse primers 5′-GCTTGAATTCATTTCATTATGATAGAAATAAC and 5′-AATTCAAGCTTTCCTATCTCATAGCCTTTTTT, which added the underlined EcoRI and HindIII restriction sites, respectively. The PCR product was digested with EcoRI and HindIII and cloned into pBluescript II SK(−) vector (Agilent Technologies, La Jolla, CA). The resulting plasmid construct was used as DNA template for PCR mutagenesis using the nucleotide analogues 8-oxo-dGTP and dPTP (17, 20). The PCR (a total volume of 25 μl) contained 2.5 units of Takara Ex TaqDNA polymerase (Clontech); 1 μM each of the forward and reverse primers used above; 2 mM $MgCl_2$; 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 400 μM each of dATP, dCTP, dGTP, and dTTP; 50 μM 8-oxo-dGTP; and 10 μM dPTP. After 42 cycles (94 °C for 1 min, 60 °C for 1.5 min, and 72 °C for 5 min), 1 μl of the amplified DNA was used in a secondary PCR in which the above conditions were used except that 8-oxo-dGTP and dPTP were omitted. The secondary PCR product was digested with EcoRI and HindIII, cloned into the T7Select10-3 phage display system, and packaged into bacteriophage according to the manufacturer's instructions (Novagen, Madison, WI).

Selection of CMG2- and TEM8-specific PAD4 Variants Using Magnetic Beads—Bacteriophage libraries were selected for CMG2-specific clones by four rounds of panning with Dynabeads TALON. Each round of panning was begun by washing 1 mg of Dynabeads TALON with TALON binding and washing buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 5 mM $MgCl_2$, 0.01% Tween 20, and 100 μg/ml bovine serum albumin) and then adding 10 μg of extracellular domain of CMG2. After incubation on a roller at 4 °C for 30 min, the beads were washed four times with the same buffer. Bacteriophage (1×10^9 particles) were then added to the mixture and incubated at 4 °C for 90 min. The beads were washed five times with the same buffer over total time periods of 10 min, 2 h, 5 h, and 20 h in the four successive panning cycles. Following the final wash in each cycle, bacteriophage were eluted from the beads with 100 μl of TALON elution buffer (150 mM imidazole, 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 5 mM $MgCl_2$, 0.01% Tween 20, and 100 μg/ml bovine serum albumin) by incubating the mixture on a roller for 10 min at 4 °C. Phage were amplified by growth in *E. coli* BLT5403 and used in the next cycle of panning. To select TEM8-specific PAD4 variants, the experimental procedures described above were conducted except that 10 μg of extracellular domain of TEM8 was used for binding to the Dynabeads.

Sequencing of DNA Inserts in T7 Bacteriophage—Bacteriophage from the fourth round of panning were picked from individual bacteriophage plaques, suspended in M9LB broth (Novagen, Madison, WI), and set at room temperature for 2 h to allow bacteriophage particles to diffuse from the agar. EDTA was then added to the bacteriophage suspension to give a final concentration of 10 mM, and the suspension was heated at 65 °C for 10 min. One microliter of the sample was used for PCR amplification in reactions containing 2.5 units of Takara Ex TaqDNA polymerase (forward primer, 5′-TAAGTACGCAATGGGCCACG3′; reverse primer, 5′-AACTCAGCGGCAGTCTCAAC3′) with PCR conditions as described above, except the annealing temperature was 50 °C. The PCR product was sequenced by dideoxy-mediated sequencing reactions.

Construction of PA Ile-656 and PA Glu-654 Mutants—PA variants were created by inserting oligonucleotide cassettes into plasmid pYS54, a PA expression vector having the silent restriction sites StuI and XhoI at positions corresponding to amino acids 650–652 and 665–667, respectively (17). To obtain all 19 amino acid substitutions of either Ile-656 or Glu-654, oligonucleotides were synthesized having the sense strand sequences 5′-TGAAGATACTGAAGGCCTTAAAgaaGTTataAATGACAGATATGATATGTTGAATATCTCGAGTTTACGGCAAGA-3′ and antisense strand sequences 5′-TCTTGCCGTAAACTCGAGATATTCAACATATCATATCTGTCATTtatAACttcTTTAAGGCCTTCAGTATCTTCA3′, where either (but not both) the Glu-654 codon gaa or the Ile-656 codon ata in the sense strand and the corresponding codons in the antisense strands (all shown in lowercase) were fully randomized. The StuI and XhoI sites of the oligonucleotides are underlined. Additionally, mutants having R659S/M662R substitutions plus all 19 amino acid substitutions of Glu-654 were made in a similar way by including the Ser-659 and Arg-662 codons in the oligonucleotides described above. In each case, double strand cassettes were obtained by heating the mixture of sense and antisense strands at 100 °C for 10 min and allowing the mixture to cool slowly to room temperature. The resulting products were digested with StuI and XhoI and ligated into pYS54 digested with the same restriction endonucleases, and the ligation mixtures were transformed into *E. coli* XL-1 Blue. Plasmid DNAs isolated from individual clones were sequenced to select all 20 amino acid substitutions.

Construction of PA Proteins with PAD4 Substitutions and Having a Modified Furin Site—To prepare PA proteins having substitutions in residues identified as being involved in binding to CMG2 and TEM8 (Ile-656 etc.) and also an uncleavable furin site, the pYS54 constructs described above were digested with PstI and HindIII, and the intervening region was replaced with the corresponding PstI to HindIII fragment isolated from a plasmid encoding PA-U7 (12), in which the ^{164}RKKR^{167} sequence is replaced by PGG. The doubly substituted PA proteins derived from PA I656V and PA I656Q were accordingly named PA-U7 I656V and PA-U7 I656Q, respectively. In a similar way, altered furin site sequences were inserted into constructs having multiple substitutions, including the PA E654T/R659S/M662R protein (abbreviated PA TSR). In this case, alternative furin site sequences used included those from PA-U7 (as above) and PA-L1, the latter being one cleaved by matrix metalloproteases (11). The doubly mutated PA proteins derived from PA TSR were accordingly named as PA-U7 TSR and PA-L1 TSR.

Expression and Purification of PA Proteins—Plasmids encoding individual constructs described above were transformed into the nonvirulent *B. anthracis* strain BH450 or BH460, and transformants were grown in FA medium with 10 μg/ml kanamycin for 15 h at 37 °C (25). PA proteins were concentrated from culture supernatants and purified by chromatography on a MonoQ column (GE Healthcare) by methods described previously (25, 28).

Measurement of PA Affinity for Receptors—Schild plot analyses were used as described previously (6, 29) to determine the affinity (apparent K_d) of PA variant proteins for cellular receptors. Briefly, the nontoxic PA-U7, PA-U7 I656Q, PA-U7 I656V, and PA-U7 TSR proteins were used as competitors to block the toxicity of wild type PA to CHO cells. Multiple PA dose-response cytotoxicity assays were done, each in the presence of a different fixed concentration of the competitor. The midpoints on the dose-response curves (*Ti*) were plotted against the competitor concentration. *Ti* is the midpoint of the dose-response curve obtained at a particular fixed competitor concentration. *To* is the value of *Ti* with no competitor added. The intercept of the resulting line at the point where $\log((Ti/To) - 1) = 0$ identifies the competitor concentration equal to the apparent K_d value, a measure of the affinity of the competitor for the receptor used by the toxin to produce toxicity.

Cells and Culture Media—Parental wild type CHO cells (CHO WTP4) and the PA receptor-expressing CHO CMG2-C4 and CHO TEM8-T4 cells, which overexpress CMG2 and TEM8, respectively, are as described previously (26, 30). CHO cells were grown in minimum essential medium α with 5% FBS, 2 mM glutamine, 5 mM HEPES, pH 7.4, and 50 μg/ml gentamicin, with or without 300 μg/ml hygromycin B. HeLa cells were cultured in Dulbecco's modified Eagle's medium with 10% FBS, 2 mM glutamine, and 50 μg/ml gentamicin. MEF cells were isolated from embryonic day 13.5 embryos of the wild type, CMG2$^{-/-}$, TEM8$^{-/-}$ and CMG2$^{-/-}$/TEM8$^{-/-}$ mice (described below) as described previously (6) and cultured in Dulbecco's modified Eagle's medium with 10% FBS, 2 mM glutamine, and 50 μg/ml gentamicin. MEF cells were passaged at 85–90% confluence by splitting 1:4 and used within the first six passages.

Cytotoxicity Assays of Cultured Cells—Cells were plated at 10,000 cells/well in 96-well plates and cultured for 24 h before treatment. PA proteins, combined with 100 ng/ml FP59 for CHO cells and with 400 ng/ml FP59 for MEF cells, were added to cells to a final volume of 200 μl/well. Cell viability was assayed 48 h after treatment by replacing the medium with 50 μl of medium containing 2.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Medium was removed after 1 h of incubation at 37 °C, and the blue pigment produced by viable cells was dissolved in 50 μl/well of 0.5% (w/v) SDS, 25 mM HCl in 90% (v/v) isopropyl alcohol. Plates were vortexed, and oxidized MTT was measured as A_{570} using a microplate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA). Results were analyzed with Prism software (GraphPad Software Inc., San Diego, CA) as percentage viability of control wells containing FP59 without PA. EC_{50} values were determined by nonlinear regression sigmoidal dose-response analysis with vari-

able slopes. Each assay was performed three times, and data from a representative assay are shown.

Lethality of PA Variants for Receptor-deficient Mice—The CMG2$^{-/-}$ and TEM8$^{-/-}$ C57BL/6 mice used here were described previously (6, 8). These mice and littermate control mice (all 8–10 weeks old) were challenged intraperitoneally with two doses of PA or PA variants along with FP59 (in 0.5 ml of PBS; doses indicated in the tables and figures) with a 2-day interval. Mice were observed for signs of malaise twice daily for 1 week following injection. All mouse studies were carried out in accordance with protocols approved by the NIAID, National Institutes of Health, Animal Care and Use Committee.

Tumoricidal Activity of PA Variants—Athymic nude mice were injected intradermally with 1×10^7 HeLa cells/mouse, and tumor xenografts were allowed to grow to 0.05% of body mass. Tumor-bearing mice were randomized into groups and injected intraperitoneally at intervals of 2 or 3 days, as indicated in the figures, with six doses of PBS or the engineered toxins. Body weights and tumor masses were measured at 2- or 3-day intervals. Tumors were measured with digital calipers (FV Fowler Company, Inc., Newton, MA), and tumor weights were estimated with the length, width, and height tumor dimensions using the formula, tumor weight (mg) = ½(length in mm × width in mm^2) or ½(length in mm × width in mm × height in mm).

Author Contributions—K.-H. C. and S. H. L. designed the study and wrote the paper. S. L., S. M.-R., Y. Z., and T. H. B. collaborated on the *in vivo* lethality of PA variants to CMG2 and TEM8 knockout mice and tumoricidal activity of PA variants to HeLa xenografts in mice. R. F. contributed to purification of proteins used in the studies. C. E. L. collaborated in the PA structural analysis. All authors approved the final version of the manuscript.

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