INFECTION AND IMMUNITY, Feb. 1994, p. 579–588
0019-9567/94/$04.00+0

Vol. 62, No. 2

Analysis of Toxinogenic Functions Associated with the RTX Repeat Region and Monoclonal Antibody D12 Epitope of *Escherichia coli* Hemolysin

GAIL E. ROWE, SHAHAIREEN PELLETT, AND RODNEY A. WELCH*

Department of Medical Microbiology and Immunology, University of Wisconsin Medical School, Madison, Wisconsin 53706

Received 29 September 1993/Returned for modification 29 October 1993/Accepted 29 November 1993

Amino acids (aa) 550 through 850 of the *Escherichia coli* hemolysin (HlyA) contain sequences important for several steps in cytolysis. These include the Ca^{2+}-binding glycine-rich tandem repeats recognized by the monoclonal antibody A10, the putative HlyC-dependent acylation site that corresponds to the monoclonal antibody D12 epitope, and the erythrocyte specificity domain which confers erythrolytic activity to the *Pasteurella haemolytica* leukotoxin. To further investigate the toxinogenic functions associated with this region of HlyA, we constructed mutants in the *hlyA* sequences coding for the repeat region and the D12 epitope. Mutants were analyzed for anti-HlyA antibody reactivity, cytolytic activities, target cell binding, Ca^{2+} requirements, and virulence. The D12 epitope was mapped to aa 673 through 726, with portions of the epitope both amino terminal and carboxy terminal to aa 700. This region was necessary, but not sufficient, for toxin binding to erythrocytes. A substitution at aa 684 resulted in loss of the D12 epitope, while cytolytic activity was retained. The nature of the D12 epitope and its associated functions are discussed. The A10 epitope mapped to residues 745 through 829, corresponding to repeats 4 through 11. Insertions within the glycine-rich repeats resulted in mutant forms of HlyA which retained A10 reactivity but required increased Ca^{2+} for lytic activity. These in vitro effects on cytolysis corresponded to a significant decrease in HlyA-mediated virulence in mice. HlyA from one insertion mutant was able to associate with leukocyte membranes under conditions that were Ca^{2+} deficient for cytolysis. The role of the glycine-rich repeats and Ca^{2+} in HlyA activity are discussed.

The *Escherichia coli* hemolysin (HlyA) is a 110-kDa polypeptide and the prototype for a family of gram-negative-bacterial, Ca^{2+}-dependent, pore-forming cytolysins known as the RTX toxins. The crucial steps leading to HlyA-induced cytolysis include the HlyC-dependent acylation of HlyA (20), Ca^{2+}-dependent activation of the toxin (35), toxin interaction with the target cell membrane (3, 5, 13), transmembrane toxin insertion (3, 13), and cation-selective pore formation (2, 3, 29) leading to osmotic swelling and cytolysis (3). The identification of HlyA domains involved in the steps of cytolysis is paramount for the study of the function of this important family of toxins.

The repeated glycine-rich (GR) 9-amino-acid (9-aa) sequence which is common to RTX toxins (41) occurs in 13 copies in *E. coli* HlyA from aa 721 through 848 (14). A monoclonal antibody (MAb), A10, which recognizes an epitope within the first 11 repeats (aa 726 through 829) has been identified (34). The GR repeats affect the efficiency of secretion of HlyA (15, 22) and are essential for hemolysis (15). Cytolytic activity of HlyA is a Ca^{2+}-dependent process (35). HlyA binds Ca^{2+} in a manner which requires the GR repeats (5). In the absence of Ca^{2+}, HlyA does not appear to bind to erythrocytes (RBCs) (4, 5). HlyA binding to RBCs also requires the *hlyC* gene product, although HlyC activity is not necessary for Ca^{2+} to bind HlyA (4, 5).

HlyC is also required for formation of an epitope recognized by MAb D12 (34). MAb D12 reacts with wild-type HlyA but not with the *hlyC*-negative form, neutralizes hemolytic activity, and recognizes an epitope between aa 626 and 726 (34). This region overlaps with the first amino-terminal GR repeat and includes epitopes recognized by nonneutralizing MAbs G3 and D1 (34). HlyC is involved in acylation of HlyA to a cytolytically active form (20). Neither the location(s) of the acylation site(s) on HlyA nor the functional significance of this modification has been determined. However, it has been postulated that modification occurs within the MAb D12 epitope and that the acylation may facilitate hemolysin association with target cell membranes through lipid-hydrophobic interactions (20).

Certain members of the RTX toxin family are cytolytic against a narrow range of target cell types. For example, the leukotoxins from *Pasteurella haemolytica* and *Actinobacillus actinomycetemcomitans* are lytic only to leukocytes from ruminants (1, 19, 39) and certain primates (42, 44), respectively. In contrast, the *E. coli* hemolysin is toxic to a wide range of cell types from humans and many other animal species (9). The investigation of the differences in target cell specificity among RTX toxins revealed discrete regions of the broadly reactive hemolysins which can confer specificity for new cell types and host species to the target-cell-limited leukotoxins (17, 28). The RBC specificity domain of the *E. coli* hemolysin is a sequence of 176 aa which is sufficient to confer erythrolytic activity to *P. haemolytica* leukotoxin (17). The RBC specificity domain spans aa 564 through 739, including the MAb D12 epitope and up to the third amino-terminal GR repeat. The functional basis for the differences in target cell specificities is not known, although variation in the ability of different toxins to bind to multifarious cell types remains the most obvious possibility.

aa 550 through 850 of HlyA contain sequences which have been indirectly implicated for several toxinogenic functions. The purpose of this study was to examine the effects of HlyA mutations within the GR repeat region and the MAb D12

* Corresponding author. Mailing address: Department of Medical Microbiology and Immunology, University of Wisconsin—Madison, 481 Medical Sciences Bldg., 1300 University Ave., Madison, WI 53706. Phone: (608) 263-2700. Fax: (608) 262-8418. Electronic mail address: welch@macc.wisc.edu.

TABLE 1. *E. coli* strains, vectors, and recombinants used

Strain or vector	Relevant characteristic(s)	Reference
E. coli strains		
DH1		24
JM101		30
CJ236		21
J198	Nonhemolytic human fecal isolate	47
XL1-Blue		8
Vectors		
pUC19		31
pACYC184		10
pBS+		40
M13mp18		31
Recombinant plasmids		
pWAM04	*hlyCABD* in pUC19	49
pSF4000	*hlyCABD* in pACYC184	47
pWAM1097	*hlyCA* in pUC19	43
pWAM1099	*hlyA* in pBS+	This study
pWAM1100	*hlyA* in pBS+	This study
pWAM826bis	*hlyA* in M13mp18	17
pWAM974	*hlyCBD* in pACYC184	17
Recombinant strains		
WAM675	DH1, pUC19 (vector)	5
WAM589	DH1, pWAM04 (HlyCABD)	49
WAM783	DH1, pSF4000 Δ*Bam*HI (HlyABD HlyC$^-$)	48
WAM802	DH1, pWAM04 Ω*hlyA Nae*I::*Cla*I linker; produces a secreted 40-kDa C-terminal HlyA	34
WAM874	DH1, pWAM802 Δ*Bam*HI (HlyC$^-$); produces a secreted 40-kDa C-terminal HlyA	This study
WAM1790	DH1, pWAM04 ΔHlyA(aa 626–673)	This study
WAM1399	DH1, pWAM04 ΔHlyA(aa 673–699)	This study
WAM1781	DH1, pWAM04 ΔHlyA(aa 701–726)	This study
WAM1160	XL1-Blue, pWAM1099	This study
WAM1215	DH1, pWAM1099 with HlyA Y696→F substitution, pWAM974 in *trans*	This study
WAM1819	DH1, pWAM1100 with HlyA E→K substitution, pWAM974 in *trans*	This study
WAM1233	DH1, pWAM1099 ΔHlyA(aa 673–745), pWAM974 in *trans*	This study
WAM1391	DH1, pACYC184 with *Xba*I site end filled (vector)	This study
WAM1527	J198, pWAM1391	This study
WAM1392	DH1, pWAM1391 Ω*Sal*I::pSF4000 *Sal*I A fragment (HlyCABD)	This study
WAM1532	J198, pWAM1392	This study
WAM1447	DH1, pWAM1392 Ω*hlyA Bgl*II::*Xba*I linker [HlyA Ω(aa829::PLQD)]	This study
WAM1528	J198, pWAM1447	This study
WAM1491	DH1, pWAM1392 Ω*hlyA Mlu*I::*Xba*I linker [HlyA Ωaa726::RSRA)]	This study

epitope on toxin function in order to better understand the relationship between discrete regions of the HlyA polypeptide and the functional requirements for toxin activity. Our results provide new information about HlyA sequence requirements and Ca^{2+} requirements for target cell binding; the HlyA sequences that affect Ca^{2+} requirements for cytolysis of different cell types; the location, nature, and function of HlyA sequences within the MAb D12 epitope; the location of other anti-HlyA MAb epitopes within this region; and the relevance of in vitro assays of HlyA activity as indicators of in vivo pathogenesis by HlyA-producing bacteria.

MATERIALS AND METHODS

Bacterial strains and recombinant DNA techniques. The bacterial strains, recombinants, and vectors used in this study are listed in Table 1. Standard recombinant DNA techniques (37) were used for mutant construction. Restriction endonucleases and DNA-modifying enzymes were obtained from New England Biolabs (Beverly, Mass.). T7 DNA polymerase was obtained from Pharmacia (Piscataway, N.J.). All plasmids were transformed into *E. coli* K-12 strain DH1 by the calcium chloride cold-shock method, unless otherwise indicated.

Recombinant strain WAM802 (34) produces two HlyA-derived polypeptides by virtue of an internal start of translation after the stop codon introduced by a *Cla*I linker insertion at the unique *Nae*I site of *hlyA*. The amino-terminal peptide is 69 kDa in size and remains cell associated, whereas the 40-kDa portion of HlyA contains the carboxy-terminal secretion signal and is exported to the culture supernatant. WAM874 was constructed by digesting plasmid DNA from WAM802 with *Bam*HI and ligating the sticky ends of the resultant A fragment, creating a derivative of WAM802 that is devoid of a functional *hlyC* gene. The recombinant plasmids pWAM1099 and pWAM1100 are derivatives of pWAM1097 (*hlyCA* in pUC19) (43) in which the *Bam*HI A fragment was cloned into the *Bam*HI site of pBS+ (Stratagene, La Jolla, Calif.) to generate plasmids containing the *hlyA* gene. *hlyA* is in the same orientation as *lacZ* in pWAM1099 and in the opposite orientation in pWAM1100. WAM1160 is *E. coli* XL1-Blue transformed with pWAM1099. This strain was used for preparing a single-stranded DNA template of *hlyA* for oligonucleotide-

mediated site-directed mutagenesis by methods specified by Stratagene. Single-stranded DNA was rescued by using Stratagene's interference-resistant helper phage VCSM13 and transfected into *E. coli* CJ236 to produce a uracil-containing single-stranded template (U template). Alternatively, U template DNA was prepared from transfection of CJ236 by M13mp18 harboring the *hlyA* gene (pWAM826bis) (17). WAM1215, WAM1233, WAM1399, WAM1781, and WAM1790 were created by in vitro site-directed mutagenesis using the methods of Kunkel (21) with the U template described above and mutagenic oligonucleotides purchased from the University of Wisconsin Biotechnology Center or synthesized in our laboratory on an Applied Biosystems 391A DNA synthesizer. The mutagenic oligonucleotide sequences (5′ to 3′, antisense; with base pair numbering as in reference 14, the substituted nucleotides underlined, and Δ indicating a deleted sequence) and respective recombinant strains are as follows: 3418 TTCATAGCTCCGAAATTGCGT (WAM 1215), 3564 CTATATGCTCATCACCA (WAM1233), 3355 ACTTCCTGTAAAACCTTAACΔ 3197 GGCAGATAAA AAGACCT (WAM1790), 3436 CCATTGAATATGAGTG AATTCΔ 3336 ATCACCACCAAGTACACG (WAM1399), and 3517 CTGCCAAAAAACTTGTCGGCΔ 3416 ATAA CTCCGATATTGCGT (WAM1781). The oligonucleotide used to construct strain WAM1233 produced an in-frame deletion of bases 3555 through 3335. Mutations were confirmed by the dideoxy sequencing method (38) using a Sequenase enzyme kit (U.S. Biochemical, Cleveland, Ohio). For WAM1215 and WAM1233, mutant HlyA was expressed in *trans* to the *hlyCBD* genes (pWAM974) (17). To construct strains WAM1399, WAM1781, and WAM1790, the altered regions of *hlyA* were subcloned from M13mp18 into the entire hemolysin operon by replacing the *Bss*HII-*Nco*I B fragment or *Bss*HII-*Bgl*II B fragment of pWAM04 (wild-type hemolysin operon) (49) with the same fragment from the mutant. The restriction enzyme pattern for each subcloned recombinant strain was confirmed by digestion with at least two different multisite enzymes.

Strain WAM1819 was generated by the following procedure. pWAM1100 was subjected to NH_2OH mutagenesis by standard methods (32). The mutation was identified by reciprocal exchange of the *Bpu*1102I-*Bgl*II fragment between the mutant and parental pWAM1100 plasmids followed by DNA sequence analysis. The glutamic acid at residue 684 was changed to a lysine by virtue of a C-to-T transition (antisense) at bp 3369.

Strain WAM1391 was made by end filling and blunt-end ligation at the *Xba*I site of pACYC184. This modified plasmid was used as the recipient DNA for the *Sal*I A fragment of pSF4000 (the entire hemolysin operon) to create WAM1392. WAM1447 and WAM1491 were constructed by cutting plasmid DNA from WAM1392 at the *Bgl*II and *Mlu*I sites, respectively, then end filling and inserting an 8-base *Xba*I linker. Mutations were confirmed by DNA sequencing. The resulting sequence includes the in-frame insertions of proline, leucine, glutamine, and aspartic acid residues at the *Bgl*II site and arginine, serine, arginine, and alanine at the *Mlu*I site. The nonhemolytic fecal isolate J198 was used for virulence studies because *E. coli* K-12 strains cannot establish an infection in mice (47). Plasmid DNAs from WAM1391, WAM1392, and WAM1447 were transformed into J198 by electroporation using a Gene Pulser from Bio-Rad (Richmond, Calif.) according to the manufacturer's protocol to create WAM1527, WAM1532, and WAM1528, respectively.

Culture conditions and hemolysin preparation. Luria-Bertani or YT medium (37) was used for liquid cultures or for 1.5% agar-based media. All strains were grown at 37°C. Antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.) and used at concentrations of 100 μg of ampicillin, 20 μg of chloramphenicol, and 20 μg of tetracycline per ml. Hemolysin was obtained from liquid (Luria-Bertani) cultures at late log phase. Cells were removed by centrifugation and filtration as previously described (46).

Immunoblot analysis. Hemolysin was precipitated from cell-free culture supernatants in 10% trichloroacetic acid at 4°C overnight, pelleted by centrifugation, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (46). Separated proteins were transferred electrophoretically to nitrocellulose membranes (46) and probed with monoclonal or polyclonal antibodies by the methods described elsewhere (4). Murine anti-HlyA MAbs D12, A10, G3, and D1 and rabbit anti-HlyA polyclonal antiserum have been described previously (7, 34, 46).

Hemolysis assay. Hemolytic activity of cell-free culture supernatants was assessed as previously described (15). Briefly, 200 μl of culture supernatant was incubated with 2% washed sheep RBCs in 0.85% saline plus 20 mM $CaCl_2$ in a total volume of 1 ml for 20 min at 37°C. Unlysed cells and cell membranes were pelleted by centrifugation, and the amount of hemoglobin released into the supernatant was determined spectrophotometrically at A_{540}. The percentage of maximum hemolytic activity was determined by dividing the A_{540} of hemoglobin released from a sample by the A_{540} of hemoglobin released from 1 ml of 2% washed RBCs by hypotonic lysis in distilled water.

Cytotoxicity to leukocytes. Leukolytic activity of cell-free culture supernatants was measured by a chromium-release assay as described elsewhere (16). Briefly, bovine lymphoma cells (BL3) were washed and incubated with 50 μCi of [^{51}Cr]sodium chromate per ml of cell suspension for 1 h at 37°C, with rocking. Cells were then washed to remove unincorporated ^{51}Cr. Two hundred thousand cells in 100 μl of tissue culture medium were incubated with 200 μl of cell-free bacterial culture supernatant for 1 h at 37°C in 5% CO_2. Unlysed cells and cell membranes were pelleted by centrifugation. The leukolytic activity of hemolysin was measured as the amount of ^{51}Cr released in 100 μl and counted (counts per minute) in a gamma counter. The leukolytic activity of hemolysin was calculated by the following formula: percent activity = (mean number of counts per minute of test sample − mean number of counts per minute in spontaneous release)/(mean number of counts per minute in maximum release − mean number of counts per minute in spontaneous release), where maximum and spontaneous releases are defined as counts per minute released by addition of 200 μl of 1 N HCl or RPMI, respectively. Spontaneous release varied among experiments, but counts were typically less than 10% of maximum release.

Target cell binding. The abilities of mutant forms of HlyA to bind to target cells were assessed by a modification of the immunoblot technique of Boehm et al. (4). Binding to RBCs was determined by incubating cell-free bacterial culture supernatants with washed RBCs as for the hemolysis assay described above. Unlysed cells and cell membranes were pelleted by centrifugation and washed in 1 ml of saline with 20 mM $CaCl_2$ three times at 12,000 × *g* for 2 min. The washed pellet was solubilized in SDS-PAGE sample buffer, heated for 15 min at 37°C, separated by SDS-PAGE, transferred to nitrocellulose, and probed with rabbit anti-HlyA polyclonal antiserum, as described above. Binding of hemolysin to leukocytes was detected by incubating bacterial culture supernatants with BL3 cells as for a chromium-release assay, except that ^{51}Cr was omitted from the assay and experiments were performed at 0

to 4°C as well as 37°C. Unlysed cells and cell membranes were pelleted, washed, solubilized, and analyzed by SDS-PAGE and immunoblots by the same procedure as that for RBCs.

Ca^{2+} titration assays. The Ca^{2+} concentration of bacterial culture supernatants was 0.1 mM, as measured by an ion selective electrode and digital ionalyzer according to the manufacturer's instructions (Orion Research Inc., Boston, Mass.). The effect of Ca^{2+} concentration on hemolytic activity was determined by washing and resuspending RBCs in saline with various concentrations of $CaCl_2$ such that the mixture of RBCs in saline plus bacterial culture supernatant provided the desired final assay concentration of $CaCl_2$. Liquid hemolysis assays were performed as described above. A maximum value for hypotonic lysis of RBCs was obtained for each Ca^{2+} concentration. To determine the amount of Ca^{2+} required for leukolytic activity, BL3 cells were prepared and labeled in tissue culture medium, as described above. After labeling, aliquots of cells were washed and resuspended in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline (11) with various concentrations of $CaCl_2$ to provide the desired final Ca^{2+} concentration when cells were mixed with bacterial culture supernatants. Chromium-release assays were performed as described above. Maximum lysis and spontaneous release were determined for BL3 cells at each Ca^{2+} concentration.

Virulence studies. The virulence of bacterial strains was assessed in a murine model for peritonitis. Female C3HeB/FeJ mice (Jackson Laboratory, Bar Harbor, Maine) were maintained at the animal care facility of the University of Wisconsin Medical School and used in virulence studies at 6 to 7 weeks of age. Bacteria were grown to a concentration of 10^9 CFU/ml as determined by plate counts. Bacterial cells were pelleted by centrifugation, washed, and resuspended to a concentration of 8×10^9 CFU/ml in 0.85% sterile saline. Inocula were prepared in a mucin solution by methods similar to those of Marks et al. (26). Bacterial suspensions were diluted serially in saline and mixed with an equal volume of 120 mg of porcine intestinal mucin, type II (Sigma), per ml in saline. Groups of four or five mice were infected intraperitoneally with 0.25 ml of these suspensions, providing 15 mg of mucin and 10^3 to 10^9 bacteria per dose. The control group received mucin in saline without bacteria. Plate counts of the final inocula were performed to verify the actual infecting doses. Hemolysis and chromium-release assays were performed on culture supernatants to confirm the cytotoxic phenotype of bacteria used for infection. Mortality of the mice was recorded between 4 and 66 h after infection. The 50% lethal dose (LD_{50}) for each time point was determined, and the data were graphed as the log of the LD_{50} over time. Differences in virulence between bacterial strains and among virulence trials were analyzed by a two-way analysis of variance (33) of the log LD_{50} over time.

RESULTS

Immunoblot analysis of mutant forms of hemolysin. Culture supernatants from *hlyA* mutant strains were analyzed by immunoblot for reactivity with MAb D12, MAb A10 (Fig. 1), and polyclonal anti-HlyA antiserum (data not shown). In all cases, the apparent molecular size of the toxin corresponded to the predicted size of HlyA from each strain. Since MAb D12 recognizes only HlyA which has been produced in the presence of HlyC, we chose this antibody as a probe for identifying regions of HlyA that are involved in the HlyC-dependent modification to its active form (34). Likewise, because MAb A10 recognizes a region of HlyA associated with hemolytic activity and Ca^{2+} binding, we also tested the HlyA for the presence of the MAb A10 epitope.

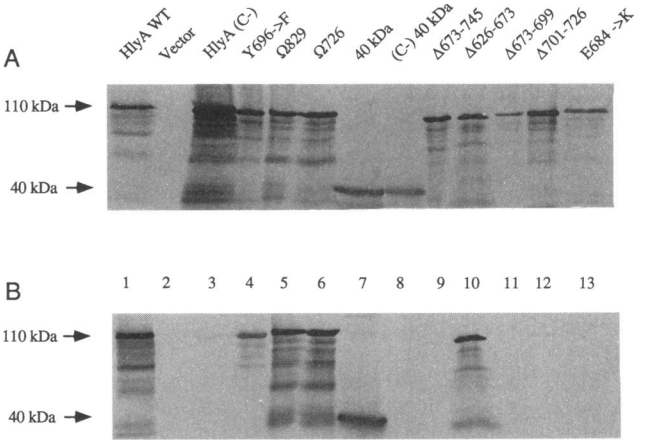

FIG. 1. Immunoblots that were used to map the epitopes of MAbs A10 (A) and D12 (B). HlyA proteins are indicated for both panels at the top. Full-length HlyA and the 40-kDa secreted polypeptide (lanes 7 and 8) are indicated on the left. Each lane contains 0.2 to 0.5 ml of cell-free culture supernatant, except for lanes 7 and 8 in both panels, which contain 4.0 and 2.5 ml, respectively.

HlyA from most strains reacted with all three antibody probes (Fig. 1). However, MAb D12 reacted with the wild-type HlyA ($HlyA_{WT}$) (WAM589) (lane 1) but not with the full-length (110-kDa) hemolysin produced in the absence of HlyC (WAM783, $HlyA_{C-}$) (lane 3), as previously reported (34). We showed previously that MAb D12 recognized the 40-kDa secreted polypeptide from WAM802 ($HlyA_{40kDa}$) but not the 69-kDa amino-terminal, cell-associated polypeptide produced by that strain (34). To demonstrate that HlyC was directly responsible for formation of the epitope within this amino-terminal region of HlyA, we compared the MAb D12 reactivities of $HlyA_{40kDa}$ and its *hlyC*-negative derivative from WAM874 ($HlyA_{(C-)40kDa}$). MAb D12 recognized $HlyA_{40kDa}$ (lane 7) but not $HlyA_{(C-)40kDa}$ (lane 8). The MAb D12 epitope was retained in HlyA from WAM1790 ($HlyA_{\Delta 626-673}$) (with aa 626 through 673 deleted) (lane 10) but not in HlyA from WAM1233 ($HlyA_{\Delta 673-745}$) (lane 9), WAM1399 ($HlyA_{\Delta 673-699}$) (lane 11), or WAM1781 ($HlyA_{\Delta 701-726}$) (lane 12) (all with an intact *hlyC* gene but with aa 673 through 745, 673 through 699, and 701 through 726, respectively, deleted). Thus, the MAb D12 epitope probably lies within aa 673 through 726 and includes regions both carboxy terminal and amino terminal to aa 700.

WAM1819 was derived from a recombinant strain that was generated by random mutagenesis of *hlyA*. HlyA from WAM1819 contains a substitution of lysine for the glutamic acid at aa 684 ($HlyA_{E684\rightarrow K}$). $HlyA_{E684\rightarrow K}$ did not react with MAb D12 (lane 13). We also examined the MAb reactivity of HlyA from strain WAM1215, in which a phenylalanine has been substituted for the tyrosine at residue 696 ($HlyA_{Y696\rightarrow F}$) by site-directed mutagenesis. Tyrosine 696 was chosen as the target for substitution because it is one of only 9 amino acids within the 53-amino-acid region containing the MAb D12 epitope that are identical in sequence alignments among eight RTX toxins (45). All eight of these toxins share the requirement for activation by a C-gene product. Of those nine amino acids, tyrosine 696 is the closest to aa 700, which is the midpoint within the 53-amino-acid region and the division point between two smaller (26- to 27-aa) regions which are

TABLE 2. Lytic activity of mutant forms of the *E. coli* hemolysin with RBCs and BL3 cells[a]

Strain	% Hemolysis	% Leukolysis
pUC19 vector, DH1 background		
WAM589 ($HlyA_{WT}$)	86 ± 15 (6)	79 ± 13 (7)
WAM675 (vector control)	1 ± 1 (6)	1 ± 4 (7)
WAM874 ($HlyA_{(C-)40kDa}$)	1 ± 0 (3)	0 ± 3 (3)
WAM1399 ($HlyA_{\Delta673-699}$)	0 ± 0 (2)	7 ± 6 (3)
WAM1781 ($HlyA_{\Delta701-726}$)	1 ± 0 (3)	0 ± 3 (3)
WAM1790 ($HlyA_{\Delta626-673}$)	1 ± 1 (2)	1 ± 0 (2)
pBS vector, *hlyCBD* on pACYC184, DH1 background		
WAM1215 ($HlyA_{Y696\rightarrow F}$)	70 ± 4 (3)	26 ± 14 (4)
WAM1233 ($HlyA_{\Delta673-745}$)	1 ± 0 (3)	0 ± 2 (2)
WAM1819 ($HlyA_{E684\rightarrow K}$)	80 ± 11 (3)	69 ± 5 (3)
pACYC184 vector with the *Xba*I site end filled, DH1 background		
WAM1391 (vector control)	1 ± 1 (6)	0 ± 3 (10)
WAM1392 ($HlyA_{WT}$)	87 ± 6 (6)	74 ± 10 (10)
WAM1447 ($HlyA_{\Omega829}$)	89 ± 2 (4)	0 ± 3 (9)
WAM1491 ($HlyA_{\Omega726}$)	87 ± 2 (5)	14 ± 22 (8)

[a] The data are means ± standard deviations, with numbers of replicates in parentheses.

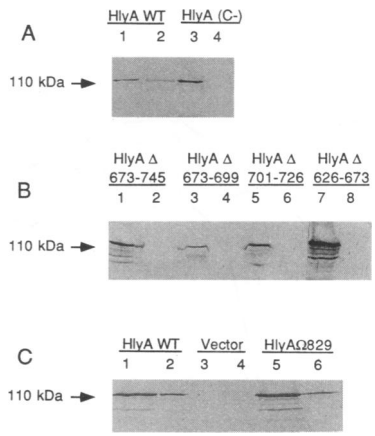

FIG. 2. Binding of HlyA to target cells detected by immunoblots probed with anti-HlyA polyclonal antiserum. HlyA proteins are indicated above each pair of lanes. Odd lanes, reaction to the amount of cell-free culture supernatant used in the assay; even lanes, HlyA associated with sheep RBCs (A and B) or BL3 cells (C). The size and location of full-length HlyA is indicated on the left. Assays contained 200 μl (A and C) or 600 μl (B) of culture supernatant. The gel in panel B was spliced between lanes 6 and 7 to remove unrelated intervening lanes.

both required for formation of the MAb D12 epitope. $HlyA_{Y696\rightarrow F}$ reacted with MAb D12 (lane 4), indicating that this substitution does not affect this epitope.

The epitopes for nonneutralizing MAbs D1, G3, and A10 were further localized by reaction with $HlyA_{\Delta626-673}$, $HlyA_{\Delta673-745}$, $HlyA_{\Delta673-699}$, $HlyA_{\Delta701-726}$, and $HlyA_{E684\rightarrow K}$. Both MAbs D1 and G3, which were previously mapped to aa 626 through 726 (34), reacted with $HlyA_{\Delta673-745}$, $HlyA_{\Delta673-699}$, $HlyA_{\Delta701-726}$, and $HlyA_{E684\rightarrow K}$ but not with $HlyA_{\Delta626-673}$ (data not shown), indicating epitopes between aa 626 and 673. The MAb A10 epitope was previously localized to aa 726 through 829 (34). Since MAb A10 reacted with $HlyA_{\Delta673-745}$ (lane 9), its epitope probably lies between aa 745 and 829. $HlyA_{Y696\rightarrow F}$ (lane 4) and $HlyA_{E684\rightarrow K}$ (lane 13) both reacted with MAb A10. The insertions within the first repeat (WAM1491, $HlyA_{\Omega726}$) (lane 6) or between the 11th and 12th repeats (WAM1447, $HlyA_{\Omega829}$) (lane 5) did not alter the phenotype of HlyA on immunoblots.

Erythrolytic and leukolytic activity of HlyA mutants. The effects of mutations in the sequences coding for the activation (MAb D12 epitope) and Ca^{2+}-binding (repeats) regions of HlyA on in vitro lytic activity of HlyA with RBCs and leukocytes were examined by a liquid hemolysis assay and a chromium-release assay, respectively. Results are expressed as percentages of maximal lysis in Table 2. In most cases, alterations in HlyA affected activity against both cell types similarly. $HlyA_{(C-)40kDa}$, $HlyA_{\Delta626-673}$, $HlyA_{\Delta673-745}$, $HlyA_{\Delta673-699}$, and $HlyA_{\Delta701-726}$ were unable to lyse sheep RBCs or BL3 cells. The small amounts of hemolysis observed with some of these strains were comparable to that seen with the vector control strain (WAM675). Similarly, the low levels of leukolysis seen for $HlyA_{\Delta626-673}$ and $HlyA_{\Delta673-699}$ were close to that produced by the vector control. $HlyA_{Y696\rightarrow F}$ and $HlyA_{E684\rightarrow K}$ were lytic to RBCs and BL3 cells, although cytolysis by $HlyA_{Y696\rightarrow F}$ was decreased against both cell types.

A very different pattern of cytolysis was seen for mutant strains with insertions in the repeat region of HlyA. $HlyA_{\Omega726}$ and $HlyA_{\Omega829}$ showed wild-type toxicity against RBCs but exhibited little or no lysis against BL3 cells. The variability among chromium-release trials with $HlyA_{\Omega726}$ was unusually high, reflecting two trials in which toxicity was moderately affected (56 and 41% of maximum lysis) and six trials in which leukolysis was 6% of maximum or less.

Binding of nonlytic hemolysin mutants to target cells. Boehm et al. (5) showed that a functional *hlyC* gene is required for HlyA to bind to RBCs. Since *hlyC* is also required for the formation of the MAb D12 epitope, we tested the hypothesis that deletion of the amino acid sequence containing the D12 epitope from HlyA would prevent HlyA from binding to RBCs. The RBC-binding capacities of nonhemolytic $HlyA_{\Delta626-673}$, $HlyA_{\Delta673-745}$, $HlyA_{\Delta673-699}$, and $HlyA_{\Delta701-726}$ were examined by immunoblot (Fig. 2A and B). To be certain that any RBC-associated HlyA was detected, the binding assay was modified to accommodate a larger volume of culture supernatant from some strains, as described in the figure legend. $HlyA_{WT}$ (Fig. 2A, lanes 1 and 2) binds to RBCs, but $HlyA_{C-}$ does not (Fig. 2A, lanes 3 and 4), as previously reported. No toxin association with RBCs was detected for HlyA missing the MAb D12 epitope ($HlyA_{\Delta673-745}$, $HlyA_{\Delta673-699}$, or $HlyA_{\Delta701-726}$) (Fig. 2B, lanes 1 through 6). Surprisingly, our data show that $HlyA_{\Delta626-673}$, which retains the MAb D12 epitope, was also unable to associate with RBC membranes (Fig. 2B, lanes 7 and 8).

$HlyA_{\Omega829}$ showed high levels of activity in the hemolysis assay but none in the chromium-release assay (Table 2). We tested the hypothesis that the loss of leukotoxicity is attributable to the inability of $HlyA_{\Omega829}$ to bind to BL3 cells. Figure 2C shows that $HlyA_{\Omega829}$ (lanes 5 and 6) was able to bind to BL3 cells with an efficiency comparable to that of $HlyA_{WT}$ (WAM1392) (lanes 1 and 2). The results were the same when the assay was performed at 0 to 4°C (data not shown), indicating that the observed association of HlyA with BL3 cells is probably not due to pinocytosis of the toxin by leukocytes.

Restoration of leukolytic activity by increased Ca^{2+} concentration. $HlyA_{\Omega726}$ and $HlyA_{\Omega829}$ contain insertions within the Ca^{2+}-binding GR repeat region. The concentration of Ca^{2+} in the chromium-release assay is 0.2 mM, whereas the final

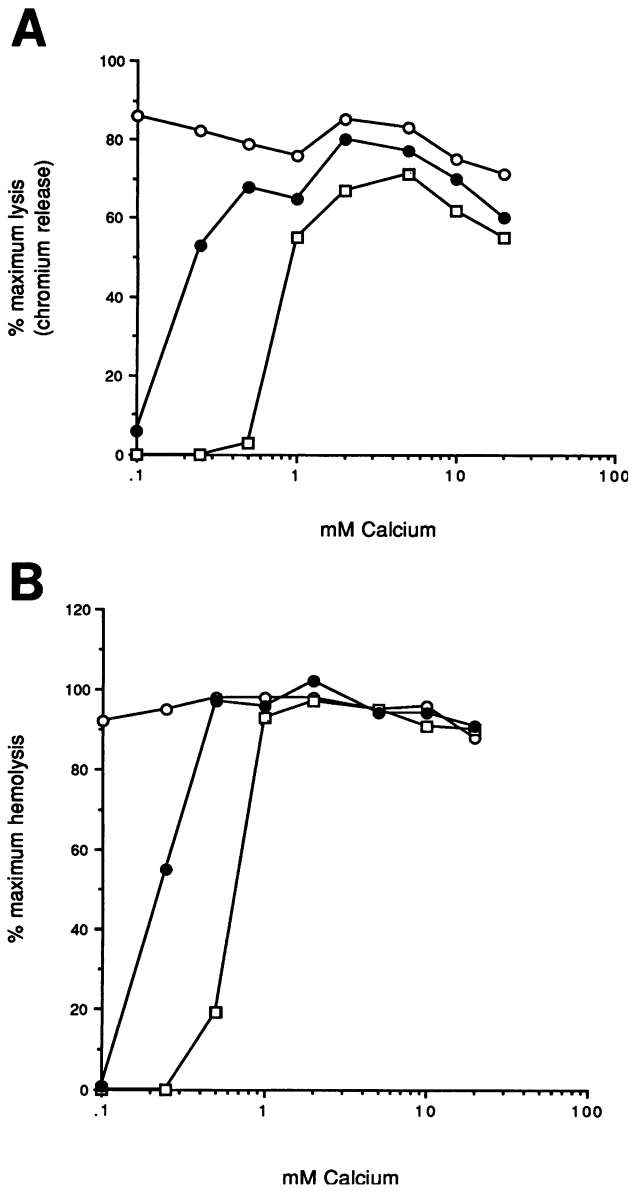

FIG. 3. Titration of Ca^{2+} required for cytolytic activity by mutant forms of HlyA. $HlyA_{WT}$ (open circles), $HlyA_{\Omega726}$ (filled circles) and $HlyA_{\Omega829}$ (squares) were tested for cytolytic activity against BL3 cells in a chromium-release assay (A) and against sheep RBCs in a liquid hemolysis assay (B) with the final concentrations of Ca^{2+} indicated on the *x* axis. Activity is reported as the percent chromium (A) or hemoglobin (B) released relative to maximum release by 1 N HCl (A) or distilled water (B). Culture supernatant from the vector control strain (WAM1391) induced no chromium release above spontaneous release and less than 0.5% of maximal hemoglobin release.

concentration of Ca^{2+} in a hemolysis assay is 16.6 mM. To determine whether the loss of leukolytic activity by $HlyA_{\Omega726}$ and $HlyA_{\Omega829}$ reflected an increased requirement for Ca^{2+}, we performed the chromium-release assay at a range of Ca^{2+} concentrations of 0.1 to 20 mM. Each strain was tested at least twice. The results from one representative experiment are shown in Fig. 3A. $HlyA_{WT}$ (WAM1392) was fully active with as little as 0.1 mM $CaCl_2$. $HlyA_{\Omega726}$ gave negligible leukolytic activity at this Ca^{2+} concentration but achieved 53% of maximum activity with 0.25 mM Ca^{2+}. When $HlyA_{\Omega726}$ was tested at 0.2 mM (the concentration present in our standard chromium-release assay) and 0.23 mM Ca^{2+}, chromium release was 10 and 35%, respectively (data not shown). $HlyA_{\Omega829}$ was barely leukolytic (3%) below 1 mM Ca^{2+} but gained activity at higher Ca^{2+} concentrations.

To determine whether increased Ca^{2+} concentration could overcome a decrease in leukolytic activity by HlyA with a mutation outside the repeat region, we tested cytolytic activity of $HlyA_{Y696\rightarrow F}$ in 0.1 to 20 mM Ca^{2+}. Although $HlyA_{Y696\rightarrow F}$ was both erythrolytic and leukolytic, it was less active against BL3 cells than RBCs (Table 2). Furthermore, the substitution mutation occurs in the same general region of HlyA as the insertion mutations ($HlyA_{\Omega726}$ and $HlyA_{\Omega829}$) but outside the GR repeats. Leukolytic activity of $HlyA_{Y696\rightarrow F}$ was not affected by this range of Ca^{2+} concentration (data not shown), suggesting that increased Ca^{2+} requirements are not a common phenotypic response to mutations outside the repeat region.

Effect of Ca^{2+} concentration on erythrolytic activity. To determine whether the erythrolytic and leukolytic activities of HlyAs from $HlyA_{\Omega726}$ and $HlyA_{\Omega829}$ were affected similarly by Ca^{2+} concentration, we tested for hemolytic activity with 0.1 to 20 mM Ca^{2+} (Fig. 3B). As in the chromium-release assay, $HlyA_{WT}$ was fully active at 0.1 mM $CaCl_2$, and $HlyA_{\Omega726}$ required greater amounts of Ca^{2+} for activity. $HlyA_{\Omega829}$ showed modest amounts of hemolysis (19%) at 0.5 mM Ca^{2+}, with peak activity at 2 mM. Hemolytic activity of $HlyA_{Y696\rightarrow F}$ was not altered by Ca^{2+} concentrations of 0.1 to 20 mM (data not shown).

Virulence of a mutant which requires more Ca^{2+} for cytolytic activity. Studies of the molecular mechanisms of pathogenesis by RTX toxins typically employ molecular manipulation of toxin structure followed by in vitro assessment of resultant changes in toxin function. Researchers do not routinely examine whether information gleaned from an in vitro system is pertinent to the toxin's contribution to virulence in vivo. Since the in vitro cytolytic activity of $HlyA_{\Omega829}$ was compromised at Ca^{2+} concentrations near the physiological concentration of free Ca^{2+} (1 mM) (12), we tested whether the alteration of HlyA affected virulence of this strain in vivo. We compared the virulence of the $HlyA_{\Omega829}$-producing strain (WAM1447) with that of strains which produce no hemolysin or the wild-type toxin in a murine model of peritonitis. We transformed the nonhemolytic fecal isolate J198 with plasmid DNA from each of the strains WAM1447, WAM1391, and WAM1392. The resultant strains are designated WAM1528 ($HlyA_{\Omega829}$), WAM1527 (vector control), and WAM1532 ($HlyA_{WT}$), respectively (Table 1). Culture supernatants from these constructs had the same phenotype for in vitro cytolysis regardless of background strain (DH1 or J198) or target cell host species (sheep RBCs and BL3 cells or freshly isolated murine RBCs, splenocytes, and peritoneal exudate cells) (data not shown).

Differences in LD_{50} were highly variable, depending on the time after infection that data were recorded (Fig. 4). Therefore, we assessed lethality throughout a time course. Mice that received mucin in saline alone showed no sign of morbidity throughout the duration of the experiment (data not shown). Higher doses (1 to >3 log units) of WAM1528 than WAM1532 were required to confer lethality at every time point (Fig. 4). Mice infected with WAM1528 began to die later after infection (18 versus 4 h) and fewer total mice died compared with mice infected with WAM1532. We controlled for host strain susceptibility by testing virulence in the outbred ICR strain and in mice of C3H lineage. HlyA has been reported to form com-

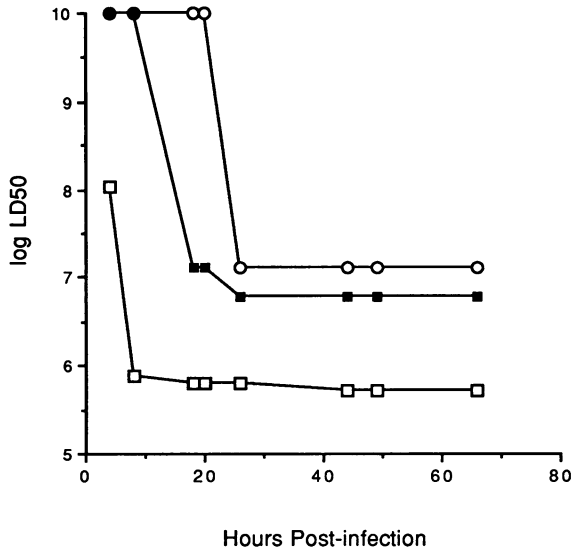

FIG. 4. Virulence of high-Ca^{2+}-requiring mutant WAM1528 (filled squares) in peritonitis of C3HeB/FeJ mice. WAM1532 (open squares) and WAM1527 (circles) produce $HlyA_{WT}$ and no toxin, respectively. The LD_{50} at each time point was calculated and is presented as log LD_{50} over time. An LD_{50} greater than 10^9 is shown as log LD_{50} = 10.

plexes with lipopolysaccharide (LPS) (6). Removal of LPS from culture supernatants by chromatography on polymyxin B Sepharose 4B resulted in concurrent loss of HlyA, as detected on Coomassie-stained gels (36). To control for the possible contribution of an LPS response to HlyA-associated virulence, the lethalities of HlyA-producing bacterial strains in LPS responder (C3HeB/FeJ) and LPS low-responder (C3H/HeJ) (27) mice were compared. Although the log LD_{50} was about 1 log unit higher for all bacterial strains tested in the LPS low-responders compared with LPS responder mice, the trends in all three mouse strains were the same (data not shown).

The statistical significance of differences in lethality among bacterial strains was assessed by a two-way analysis of variance of the log of the LD_{50} over time. Lethality of WAM1528 was significantly different from that of WAM1532 ($\alpha = 0.01$) but not significantly different at $\alpha = 0.05$ from that of the vector control strain WAM1527. The virulence of WAM1528 was tested in only one trial with each mouse strain. To assess the inherent experimental variability of this study, we compared the results of three separate virulence trials of WAM1532 and WAM1527 in ICR mice. For each trial, the log of the LD_{50} for infection with WAM1532 was subtracted from the log of the LD_{50} for infection with WAM1527 at each time point. The differences in log LD_{50} (WAM1532 minus WAM1527) over time for all three trials were not statistically significantly different from each other at $\alpha = 0.05$ by two-way analysis of variance. Therefore, we accepted with reasonable confidence the results of a single trial with WAM1528.

DISCUSSION

In this study, we made specific alterations within the MAb D12 epitope and the adjacent GR repeats to determine the contributions of these regions to certain essential steps in cytolysis by *E. coli* HlyA. Our results provide new information about the nature and function of the HlyA region within the MAb D12 epitope, structural requirements for HlyA binding to target cells, and the parameters and importance of Ca^{2+}

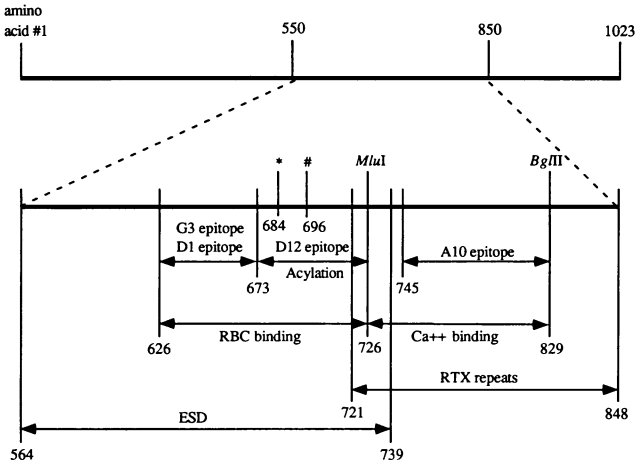

FIG. 5. HlyA functional domains relevant to this study. Numbers correspond to the 1,023-aa HlyA sequence. ESD, RBC specificity domains of *E. coli* hemolysin which confers erythrolytic capacity to nonhemolytic *P. haemolytica* leukotoxin; G3 and D1 epitopes, sequences recognized by nonneutralizing anti-HlyA MAbs G3 and D1; D12 epitope, the sequence on HlyC-modified HlyA that is recognized by the erythrolysis-neutralizing anti-HlyA MAb D12; Acylation, putative acylation or activation site which also corresponds to the D12 epitope; RBC binding, loss of detectable HlyA associated with RBCs as a result of deletion of the sequences containing the D12 epitope or the D1 and G3 epitopes; *, location of an amino acid substitution which causes loss of the D12 epitope while cytolytic activity is retained; #, location of an amino acid substitution which retains both D12 reactivity and cytolytic activity; RTX repeats, location of the 13 tandem GR 9-aa repeats, 11 of which are required for binding calcium (Ca++ binding) to HlyA. aa 726 lies within the first repeat, corresponds to the *Mlu*I restriction enzyme site of the *Xba*I linker insertion which created the higher-Ca^{2+}-requiring $HlyA_{\Omega726}$, and is the central point for overlap of several toxinogenic functional regions. A10 epitope, the sequence containing repeats 4 through 11 which is recognized by the RTX family panreactive anti-HlyA MAb A10. aa 829 corresponds to the *Bgl*II restriction enzyme site of the *Xba*I linker insertion which created the higher-Ca^{2+}-requiring $HlyA_{\Omega829}$ and lies between repeats 11 and 12.

concentration dependence for toxin function, both in vitro and in vivo.

Pellett et al. (34) showed that the erythrolysis-neutralizing MAb D12 recognizes an epitope between HlyA aa 626 and 726 which is present in active HlyA but not in the inactive form produced in the absence of HlyC. In the present study, we show that HlyC causes the MAb D12 epitope formation by the reactivity of MAb D12 with $HlyA_{40kDa}$ but not its HlyC-negative derivative. Presumably, this fragment contains the substrate for HlyC-dependent acylation (20). Alternatively, the epitope may be formed when acylation occurs elsewhere in HlyA. However, this would require multiple acylation sites which induce the formation of this epitope such that deletions of several different portions of HlyA, as were used to map the MAb D12 epitope, never eliminate all the acylation targets.

The localization of the MAb D12 epitope is extremely important for directing our future research of the HlyC-dependent activation process. By immunoblot analysis of mutations within the MAb D12 epitope, we have localized the putative acylation target to 53 aa surrounding aa 700 (Fig. 5). Consequently, we have reduced the size of our target for further mutant analyses by half. We also showed that both of two smaller regions (26 and 27 aa) on either side of aa 700 are required for formation of the MAb D12 epitope. The substi-

tution mutation at aa 684 and the deletion of aa 701 through 726 each prevented reactivity by MAb D12, suggesting that the epitope may be linear and defined by at least aa 684 through 701. However, these data could also indicate a conformational epitope. As such, the epitope must either contain elements both amino terminal and carboxy terminal to aa 700 or else be dependent on one domain for formation of the epitope in the other domain. Surprisingly, the Glu-to-Lys substitution at aa 684 lacked MAb D12 reactivity but retained cytolytic activity. These data suggest that aa 684 may be part of the domain which forms the MAb D12 epitope and not one of the residues actually involved in HlyC-mediated activation. A substitution of Phe for the Tyr at aa 696 did not prevent MAb D12 reactivity in spite of the location of this residue between aa 684 and 701. This result suggests that the MAb D12 epitope is not linear. However, we recognize that this is a conservative substitution and may not be sufficiently different from the wild type to prevent MAb D12 reactivity. Our data indicate that the MAb D12 epitope may be difficult to define specifically as a smaller, linear sequence. Analyses of other random and site-directed substitution mutations which cause the loss of the MAb D12 epitope are in progress.

Previously, the epitope for hemolysis-neutralizing MAb D12 was mapped to the same 100 aa (aa 626 through 726) as nonneutralizing MAbs D1 and G3 (34). In this study, we have determined that the epitopes for D1 and G3 reside within the amino-terminal 48 aa and the D12 epitope is in the carboxy-terminal 53 aa of this region (Fig. 5). This shows a distinction between the HlyA sequences which form the epitopes for these neutralizing and nonneutralizing MAbs.

HlyC is required for formation of the MAb D12 epitope and for HlyA binding to RBCs (5). Issartel et al. (20) showed that the HlyC-dependent activation of HlyA is an acylation reaction. These authors postulated that acylation occurs within the MAb D12 epitope and facilitates toxin binding to target cells, although they did not test these hypotheses. We tested, directly, whether HlyA sequences containing the MAb D12 epitope facilitate HlyA association with RBCs. Our data show that deletion of HlyC, the region containing the MAb D12 epitope, or a stretch of 48 aa adjacent to the D12 epitope prevents RBC binding. Thus, it appears that HlyC and the region containing the MAb D12 epitope are necessary, but not sufficient, for HlyA binding to RBCs. Formation of the MAb D12 epitope, per se, cannot be the essential step for HlyA interaction with RBCs, since $HlyA_{E684\rightarrow K}$, which has lost the D12 epitope, is still hemolytic. Perhaps HlyC activity induces changes in HlyA secondary structure in the domain from aa 626 to aa 726 which facilitate HlyA binding to RBCs. It may be the same conformational change which causes the formation of the MAb D12 epitope. The substitution mutation at aa 684 may disrupt the HlyC-induced conformation of this domain enough to cause the observed loss of the D12 epitope, yet not enough to severely impede HlyA-mediated cytolysis.

The epitope recognized by MAb A10 lies within the GR repeat region (34). This MAb is an RTX pan-reactive MAb used to identify additional members of the RTX family (23). By assessing reactivity with HlyA from deletion mutants, we have now further localized the MAb A10 epitope to aa 745 through 829, including repeats 4 through 11 (Fig. 5). Amino acid insertions within the GR repeats, at aa 726 or aa 829, did not disrupt this epitope. However, these insertions within the first GR repeat ($HlyA_{\Omega 726}$) or between the 11th and 12th repeats ($HlyA_{\Omega 829}$) of HlyA resulted in a requirement for higher Ca^{2+} concentrations than that for $HlyA_{WT}$ to achieve cytolysis of either RBCs or BL3 cells. Ludwig et al. (25) reported that HlyA with deletions of one or two GR repeats required elevated levels of Ca^{2+} for hemolytic activity. However, these authors did not examine Ca^{2+}-dependent activity against nucleated cells nor the ability of the toxin to directly associate with target cells. We observed that the peak of cytolytic activity occurred at a lower calcium concentration for RBCs than for BL3 cells. However, the calcium concentration at half-maximum activity was the same regardless of cell type. Maximal restored leukolytic activity by the insertion mutants was still 10 to 25% less than wild-type activity. In contrast, wild-type levels of hemolytic activity were observed for both insertion mutants. Whether these subtle variations in cytolytic activity against different cell types have biological relevance is not known. It is significant that we showed that $HlyA_{\Omega 829}$ was able to associate with BL3 cell membranes under conditions that were Ca^{2+} deficient for cytolysis. These results suggest that Ca^{2+} may not be necessary in order for HlyA to initiate an association with leukocyte membranes.

Since detectable $HlyA_{\Omega 829}$ was associated with BL3 cells under low-Ca^{2+} conditions, the increased requirement for Ca^{2+} appears to reflect deficiency at some step required for cytolysis but subsequent to toxin association with target cells. Ca^{2+} has been reported to induce conformational changes in the *E. coli* HlyA (35) and the related *Bordetella pertussis* adenylate cyclase/hemolysin (18) that are presumed to be necessary for toxin association with target cell membranes. In accordance with our data, lower concentrations of Ca^{2+} may be sufficient to facilitate a detectable association of HlyA with certain target cells, but higher concentrations of Ca^{2+} may be required to induce the conformational changes needed for insertion or pore formation in target cell membranes.

We observed that $HlyA_{\Omega 829}$ needed more Ca^{2+} for cytolysis than did $HlyA_{\Omega 726}$. Differences in the amount of Ca^{2+} required for cytolysis may be due to either the location or the nature of the mutations tested. GR repeats 11 and 12 may be more crucial than the first repeat for the Ca^{2+}-dependent cytolytic activity of HlyA. Alternatively, it may be the nature of the disruption of secondary structure within the GR repeat region which determines the level of Ca^{2+} required to restore activity. $HlyA_{\Omega 829}$, but not $HlyA_{\Omega 726}$, includes an inserted proline residue which could significantly alter protein conformation. The high-Ca^{2+}-requiring hemolytic mutants examined by Ludwig et al. (25) were limited to large deletions in the central portion of the GR repeat domain (repeats 5 through 11). The region between aa 721 and aa 739 (the first two GR repeats) was not identified by those authors as part of the repeat domain and not examined for a role in Ca^{2+}-dependent hemolysis. We have provided a more precise definition of this phenomenon. Our data show that the first GR repeat contributes to Ca^{2+}-dependent toxin activity in spite of its divergence from the consensus sequence for an RTX repeat (14). We restored full hemolytic activity of the insertion mutants with 0.5 to 2 mM Ca^{2+}. In contrast, Ludwig et al. reported maximal hemolytic activity at 12 to 78% below that for the wild type with 5 to 100 mM Ca^{2+}. The incomplete restoration of hemolytic activity they observed may reflect elimination of HlyA sequences which are essential to toxin function but unrelated to the Ca^{2+}-dependent phenomenon. Our insertion mutations disrupt HlyA structure and function while retaining all the residues from the wild-type primary sequence.

In this study, we have shown that insertions within the GR repeat region yield moderate perturbations as evidenced by the retention of the MAb A10 epitope and the ability of the mutant HlyA to associate with target cell membranes. However, these mutations caused measurable effects on the Ca^{2+} requirements for in vitro lysis of RBCs and nucleated cells. Most importantly, the in vitro effects on cytolysis corresponded

to a significant decrease in HlyA-mediated virulence in mice. We have shown that determining the effects of molecular manipulations of HlyA by in vitro assays for cytolytic activity provides an accurate reflection of the effects on toxin-associated virulence in an animal model. On the basis of our in vitro data and studies of murine peritonitis, the infected animal host may represent an environment that is Ca^{2+} deficient for $HlyA_{\Omega 829}$ toxin activity. Our in vitro observations indicate that small differences in Ca^{2+} concentration can have important ramifications for cytolysis. We reported a high degree of variability among repetitions of the chromium-release assay for $HlyA_{\Omega 726}$. This variability can be attributed to large differences in cytolytic activity corresponding to small differences in Ca^{2+} concentration between 0.20 and 0.25 mM. These results may indicate an important role for potential Ca^{2+} fluctuations in vivo in HlyA-mediated pathogenesis.

From this study and previous analyses of hybrid toxins and mutants containing alterations within amino acids 550 through 850 of HlyA, it is clear that this is a region of HlyA important for essential toxin functions. Sequences in this region have been implicated in HlyC-dependent modification of HlyA, RBC and Ca^{2+} binding, Ca^{2+}-mediated cytolysis, and target cell specificity among different RTX toxins. Our studies of mutations within the sequences coding for the GR repeat region and the MAb D12 epitope have helped to define crucial domains for toxinogenic functions and have provided reagents for further study of toxin activity, both in vitro and in vivo.

ACKNOWLEDGMENTS

This work was funded by Public Health Service grant AI20323 and by the Romnes Fellowship from the University of Wisconsin.

We thank Tony Lobo for helpful discussions.

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