Escherichia coli Hemolysin Mutants with Altered Target Cell Specificity

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In order to understand the functional significance of HlyC-dependent acylation of the *Escherichia coli* hemolysin structural protein (HlyA), random as well as site-directed substitutions at the known regions of modification, i.e., those at lysine residues at amino acid positions 563 and 689 (HlyA_{K563} and HlyA_{K689}, respectively), were isolated. Sixteen random *hlyA* mutations were identified on the basis of a screen for loss of immunoreactivity to the hemolysin-neutralizing D12 monoclonal antibody that reacts to only HlyC-activated HlyA. These substitutions occurred at the region from HlyA_{E684} to HlyA_{Y696}. A recombinant glutathione *S*-transferase-hemolysin gene fusion encoding glutathione *S*-transferase-HlyA_{S608-T725} residues reacts with monoclonal antibody when HlyC is coexpressed with the fusion protein. Therefore, at most only 12% of the total HlyA primary sequence is needed for HlyC-facilitated acylation at the HlyA_{K689} position, and this modification can occur in the absence of the proximal HlyA_{K563} acylation site. The cytolytic activities of these HlyA mutants against sheep erythrocytes and bovine and human lymphocyte cell lines (BL-3 and Raji cells, respectively) were analyzed. HlyA_{K563} and HlyA_{K689} substitutions displayed various degrees of loss of cytotoxicity that depended on the particular amino acid replacement. An HlyA_{K563C} variant retained greater than 59 and 21% of its BL-3-lytic and erythrolytic activities, respectively, but was nearly inactive against Raji cells. An HlyA mutant with a K-to-E substitution at amino acid 689 (HlyA_{K689E}) was essentially inactive against all three cell types, whereas an HlyA_{K689R} substitution had a pattern of activity similar to that of the HlyA_{K563C} mutant. Preceding the two in vitro acylated HlyA lysines are glycines that appear to be the only amino acids conserved in alignments of these regions among the RTX toxins. Remarkably, considering the retention of cytotoxic activity by some HlyA_{K689} mutants, each of three different substitutions at the HlyA_{G688} position was relatively inactive against all three cell types tested. This suggests that HlyA_{G688} plays a significant structural role in cytotoxic activity apart from its possible participation in an HlyC activation process which presumably requires recognition of pro-HlyA structures. The related RTX toxin, the *Pasteurella haemolytica* leukotoxin structural protein (LktA), can be activated in an *E. coli* recombinant background by HlyC. In amino acid sequence alignments, LktA_{K554} is equivalent to the HlyA_{K563} position but it has an asparagine (LktA_{N684}) at the homologous HlyA_{K689} site. An LktA_{N684K} substitution possesses wild-type leukotoxin activity against BL-3 cells and does not acquire hemolytic or Raji cell cytotoxic activity. Surprisingly, both LktA_{K554C} and LktA_{K554T} substitutions retain considerable BL-3 cytotoxicity (45 and 49%, respectively), indicating that there may be additional lysines within LktA that the HlyC activation mechanism is capable of acylating. Based on these results and a comparison of amino acid sequence alignments of 12 RTX toxins, a putative consensus structure of the RTX residues necessary for HlyC activation is hypothesized.

The *Escherichia coli* hemolysin is an exotoxin capable of inducing metabolically disruptive Ca²⁺ influxes and eventual lysis of a variety of cell types from different hosts (for a review, see reference 31). Among the different pathogenic types of *E. coli*, this toxin is produced by the most common isolates that cause upper urinary tract infections in both men and women without underlying disease or anatomical complications (for a review, see reference 6). The *E. coli* hemolysin is a member of the RTX family of cytotoxins which are produced by a variety of gram-negative animal and human pathogens, including members of the following genera: *Pasteurella*, *Actinobacillus*, *Bordetella*, *Proteus*, *Morganella*, and possibly *Moraxella* (31).

Controversy exists over the presence of lipopolysaccharide in the large, >300-kDa lytic *E. coli* hemolysin complex (5, 29). It is clear, however, that the only polypeptide component is the

110-kDa product of the *hlyA* gene. In order to be cytotoxic, the HlyA protein must be modified through the poorly understood activity of a second, cotranscribed gene product, HlyC. The chemical nature of the modification was recently discovered to be an amide-linked acylation (27). The sites of acylation for in vitro modified HlyA of murine *E. coli* origin occur at lysine residues at amino acid positions 564 and 690 in HlyA (HlyA_{K564} and HlyA_{K690}, respectively) (27). It is unknown if these sites are uniformly modified in vivo. The adenylate cyclase/hemolysin of *Bordetella pertussis* shares sequence similarity to the *E. coli* hemolysin and is also acylated by an HlyC homolog (2). The in vivo adenylate cyclase/hemolysin is acylated at only a single lysine residue (CyaA_{K983}), which by sequence alignments corresponds to HlyA_{K690} (11). When the adenylate cyclase/hemolysin is expressed in a recombinant form in an *E. coli* background, the CyaA_{K680} position (equivalent to the HlyA_{K564} position) is acylated approximately 66% of the time (12). Interestingly, this form of the adenylate cyclase/hemolysin is less hemolytic than the wild-type toxin but maintains its natural cell-invasive, adenylate cyclase-mediated levels of toxicity. This suggests that in some instances, acylation

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
DH1	Background strain	17
C600	Background strain	1
JM101	Background strain	19
CJ236	<i>dut-1 ung-1 thi-1 relA1</i>	14
pSF4000	<i>hlyCABD</i> from J96 in pACYC184	32
pWAM04	<i>hlyCABD</i> subcloned from pSF4000 in pUC19	33
pWAM1100	<i>hlyA</i> from J96 in pBS+	25
pWAM1111	<i>lktA</i> from pWAM828 ^a in pBS+	This study
pWAM974	<i>hlyCBD</i> from J96 in pACYC184	25
pANN202-812	<i>hlyCABD</i> from pHLY152 in pBR322	18
pANN202-812AL5*	HlyA _{Δ238-259}	18
pANN202-812FG10*	HlyA _{K690M}	18
pANN202-312AL10-1*	HlyA _{Δrepeat 5}	18
pGEX-2T	GST fusion vector	26

* From Forestier and Welch (9).

of specific lysines of an RTX toxin may adversely affect cytotoxic activities.

In this study we examined the cytotoxic phenotypes of *E. coli* hemolysin mutants possessing substitutions at and around the known *in vitro* sites of HlyA lysine acylation. In addition, we examined the effects of substitutions at the equivalent sites in the *Pasteurella haemolytica* leukotoxin, which has significant amino acid sequence similarity with the *E. coli* hemolysin but which, unlike the broad range of susceptible target cells for the hemolysin, is toxic to only leukocytes of ruminant origin. These studies revealed that hemolysin target cell specificity can be influenced by amino acid substitutions at the acylation sites and that it is likely that the leukotoxin is acylated at a site besides those equivalent to the HlyA_{K564} and HlyA_{K680} positions.

MATERIALS AND METHODS

Strains and media. Bacterial strains and recombinant plasmids and vectors used in this study are summarized in Table 1.

Unless noted otherwise, chemicals were acquired from Sigma (St. Louis, Mo.) and formulations of media used in this laboratory were described previously (21, 25). LB and YT media were used and supplemented with appropriate antibiotics (ampicillin [100 µg/ml] and chloramphenicol [20 µg/ml]) as previously described (9).

Mutagenesis. Site-directed mutagenesis was performed essentially by the U-temple method as described before, with either *hlyA* or *lktA* recombinant subclones in M13 phage or pBS+ phagemid vectors (9, 25). Screening of potential mutated DNAs was performed by oligonucleotide hybridization with a commercially purchased enhanced chemiluminescence 3' oligonucleotide-labeling and detection system (ECL system; Amersham, Chicago, Ill.).

Random mutagenesis was performed by two different methods. NH₂OH mutagenesis was performed as described by Miller (20). Dye-buoyant density ultracentrifugation-purified pWAM1100 DNA (*hlyA* in pBS+) (25) was mixed with a solution containing 100 µl of 0.5 M KPO₄-5 mM EDTA, (pH 6.0), 200 µl of 1 M NH₂OH, and 200 µl of double-distilled H₂O and was incubated at 37°C for periods of time varying from 16 to 36 h. The treated DNA was then dialyzed against 2 liters of 10 mM Tris-HCl (pH 7.5)-1 mM EDTA (TE buffer) for 4 h at 4°C. The DNA was ethanol precipitated and resuspended in TE buffer in a volume sufficient to concentrate the DNA approximately 50-fold. The DNA was then used to transform WAM974 (DH1 with a pACYC184-based recombinant encoding *hlyCBD* [25]), and the resulting transformants were screened for altered beta-hemolytic phenotypes on sheep erythrocyte agar plates containing ampicillin and chloramphenicol. Representatives of all observed phenotypes from those with no zones of hemolysis to those with hemolysis zones two to three times greater in diameter than the wild-type zones were patched to LB agar plates containing chloramphenicol and ampicillin. The resulting cell growth after overnight incubation at 37°C was used for immunoprobings by a colony lift procedure modified from the method described in the procedures manual for the Amersham ECL detection system. Three different antibody preparations were used in this analysis. To ensure that the HlyA antigen was still being produced by potential

mutant strains, rabbit anti-HlyA serum was used and only candidates with an unaltered immunobased signal to this polyclonal reagent were chosen for further study. To detect alterations in the epitopes associated with the HlyA_{K680} acylation site and the HlyA Ca²⁺ binding domain, the murine monoclonal antibodies (MAbs) D12 and A10, respectively, were used (23, 25). Of special note is that the blocking buffer for nitrocellulose filters probed with either the polyclonal or A10 antibodies was phosphate-buffered saline (PBS) plus 0.1% Tween 20. However, filters probed with the MAb D12 were probed with PBS plus 5% powdered skim milk because the PBS-Tween blocking buffer prevents detection of D12 reactivity with the HlyC-activated form of the HlyA antigen. Development and detection of antibody reactivity were performed as described in the ECL manual. Controls for loss of reactivity of the D12 and A10 MAbs were provided by including cells from WAM783 (HlyABD) and WAM713 (HlyCA_{ΔD728-A829}BD [HlyCABD with the region D-728 to A-829 deleted from the A locus]), respectively (23). Candidate HlyA mutant proteins that were still reactive to anti-HlyA polyclonal serum but which were nonreactive to D12 and/or A10 MAbs were then subjected to immunoblotting (23) in order to examine the relative size and quantity of the mutant HlyA forms expressed. The locations of *hlyA* mutations in the candidate mutants were initially determined by isolating and purifying the *hlyA*-containing plasmid DNA and isolating a 703-bp *Bpu*1102I-*Bgl*II restriction endonuclease fragment which was then substituted for the same fragment from the original pWAM1100 in a ligation reaction. The ligated DNA was then used to transform WAM974, and transformants were reexamined for their hemolytic and antibody phenotypes. The mutations associated with the *Bpu*1102I-*Bgl*II fragment were identified by DNA sequence analysis as previously described (25).

The second random mutagenesis method, based on misincorporation of bases during PCR DNA amplification, was used with modifications (15). Purified pWAM1100 DNA was used as a template for amplification with one oligomer corresponding to bp 3022 to 3042 being combined with either of a pair of oligomers corresponding to the reverse complement of bp 3582 to 3599 or 3876 to 3890 (8). To increase the chances of misincorporation of bases, the concentration of individual deoxynucleoside triphosphates (dNTPs) in the reactions was reduced from the typical 250 µM normally used to a variety of concentrations, down to as low as 37 µM dNTPs. The amplified DNA fragments were then digested with *Bpu*1102I and either *Mlu*I with the use of the oligomer from bp 3599 to 3582 or *Bgl*II in the case of the oligomer from bp 3876 to 3890. The resultant fragments were then substituted in pWAM1100, and mutation analysis was performed as described above.

Hemolysis. The lytic capability of wild-type hemolysin and the different HlyA mutant proteins against sheep erythrocytes (hemolysis) was assessed by the recently elaborated method of Bauer and Welch (3). Briefly, this involves twofold serial dilution of toxin-containing culture supernatants, spectrophotometric measurement of the hemoglobin released after a 1-h incubation of the toxin dilutions with a 1% suspension of sheep erythrocytes, construction of a dose-response graph, and calculation of the slope of hemolytic activity using a line drawn from the first three toxin doses that gave hemolysis levels above those of the background controls.

Cytotoxicity. Two different cultured cell lines, BL-3 (bovine lymphoma) and Raji (human B-cell lymphoma), were used in ⁵¹Cr release assays of toxin cellular lysis. The growth, maintenance, and radiolabeling of these cells were performed as previously described (3). Toxin-containing filtered culture supernatants were serially diluted twofold in HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-buffered saline (0.8% NaCl-0.04% KCl-0.1% sucrose-10 mM HEPES [pH 7.4]-6 mM CaCl₂) (HBS-CaCl₂), approximately 2 × 10⁵ ⁵¹Cr-labeled target cells were added to each toxin dilution, and the mixtures were incubated for 1 h at 37°C. The unlysed cells were removed by centrifugation at 740 × g, and the amount of lysis was determined by measuring the released ⁵¹Cr in terms of counts per minute. To determine total cell lysis, cells were lysed with 0.7 M HCl, and background release of ⁵¹Cr was determined from incubation of the cells with just HBS-CaCl₂. The following formula was used to calculate percent lysis: 100 × (sample cpm - background cpm)/(total cpm - background cpm), where cpm is ⁵¹Cr release in counts per minute.

Electrophoresis and immunoblotting. The trichloroacetic acid precipitation of HlyA and LktA proteins from toxin-containing culture supernatants and sample preparation for sodium dodecyl sulfate-10% polyacrylamide electrophoresis (SDS-PAGE) were performed as described previously (3). Proteins separated by electrophoresis were prepared for immunoblotting essentially by the method of Towbin et al. (28). Nitrocellulose blots were blocked and probed with either rabbit polyclonal antibodies or murine MAbs, and antigen-antibody complexes were detected as described recently (3).

HlyA and LktA in culture supernatants were quantified as follows. Culture supernatant samples were concentrated by trichloroacetic acid precipitation, the samples were serially diluted twofold, each dilution was subjected to SDS-PAGE, and the separated proteins were stained with Coomassie brilliant blue. The intensity of the stained polypeptides present in dried gels was measured by a scanning densitometer and compared with that of serial dilutions of a known amount of commercially purified β-galactosidase run on the same gel.

RESULTS

The structural and functional significance of the HlyC-facilitated acylation of the *E. coli* hemolysin is poorly understood. The discovery by Stanley et al. (27) of in vitro acylation of HlyA_{K564} and HlyA_{K690} residues by an apparently novel mechanism combined with the localization of the HlyC-dependent anti-HlyA D12 MAb HlyA epitope between HlyA_{V673} and HlyA_{R726} (HlyA_{V673-R726}) (25) prompted our studies to better define the D12 epitope and the functional consequences of substitutions at and around the HlyA_{K564} and HlyA_{K690} positions. It is instructive to indicate that our numbering of HlyA residues differs from that of Stanley et al. because the *E. coli* sources of the *hlyA* genes differ and these genes are not entirely identical (8, 13). Consequently, the HlyA numbering for the sequence studied in Great Britain has the acylated lysines at positions 564 and 690 whereas the homologous positions reported in this work are numbered 563 and 689. The results of the mutagenesis and phenotypic studies are summarized in Tables 2 and 3. Hydroxylamine mutagenesis of the entire *hlyA* gene resulted in identification of six independent substitutions (HlyA_{E684K}, HlyA_{G688R} [occurred twice], HlyA_{G688E} [occurred twice], and HlyA_{G688K}) in which there is loss of the MAb D12 anti-HlyA reactivity without loss of the neighboring, distal A10 MAb epitope (23, 25). These results led to the isolation of additional HlyA substitutions by focusing mutagenesis efforts on just the *hlyA* sequences encoding the HlyA_{S391-A829} region. PCR-based mutagenesis resulted in 10 independent mutants lacking the HlyA D12 epitope. Of 10 PCR-generated *hlyA* mutations, 4 represented single amino acid substitutions (HlyA_{K689R}, HlyA_{K689E}, and HlyA_{T694P} [occurred twice]). The remaining 6 mutations represented double HlyA substitutions in which one of the substitutions fell within the HlyA_{S686-Y696} span and the other substitution was present 11 to 28 positions away (e.g., HlyA_{Y696C,T724A} and HlyA_{T666A,G688R}). Thus, the location of HlyA substitutions that affected loss of the HlyC-dependent D12 MAb reactivity coincided with the 12-amino-acid HlyA_{E684-Y696} region within which HlyA_{K689} represents one of the two lysines acylated in the in vitro system described by Stanley et al. (27). This is also the homologous site for in vivo acylation of the *B. pertussis* adenylate cyclase/hemolysin (11). Oligonucleotide site-directed mutagenesis was employed to isolate an HlyA_{K689C} substitution for this study and for use in thiol-derivatization experiments that will be reported elsewhere (22). This HlyA mutant protein is also no longer reactive to the D12 MAb. Lastly, the HlyA_{K690M} substitution isolated by Ludwig et al. (18) was found to be nonreactive to the D12 MAb in contrast to the wild-type HlyA encoded by the recombinant plasmid pANN202-312.

To test the hypothesis that the apparent acylation at the proximal HlyA_{K563} site is required for creation of the HlyC-dependent D12 epitope at the distal HlyA_{K689} position, two different HlyA_{K563} substitutions were isolated by oligonucleotide site-directed mutagenesis. Neither the HlyA_{K563T} nor the HlyA_{K563C} substitutions are affected in their D12 reactivity. This observation supports the earlier report that at least in *cis* configuration, the HlyA_{K689} modification associated with the D12 epitope does not require any portion of the HlyA_{M1-A626} sequence (25). We were also curious as to the HlyA structural context required for the HlyC-dependent HlyA modification and expression of reactivity to the D12 MAb. In other words, could a small portion of the HlyA polypeptide containing the HlyA_{K689} region be recognized in vivo by the HlyC activation mechanism? To initiate these studies, glutathione *S*-transferase-hemolysin gene fusions were constructed in back-

TABLE 2. Summary of *hlyA* and *lktA* mutations in this study

pWAM no. and mutant	WAM no. ^a	bp change ^b	Substitution	Mutagenesis method
<i>hlyA</i>				
2158	2160	3005 A→T 3007 A→T 3008 A→C	K563T	SDM ^c
2119	2121	3006 A→T 3007 A→G 3008 A→C	K563C	SDM
2229	1819	3371 G→A	E684K	NH ₂ OH ^d
1911	1918	3381 G→A ^e	G688R	NH ₂ OH
1912	1919	3382 G→A ^e	G688E	NH ₂ OH
1913	1920	3381 G→A 3382 G→A	G688K	NH ₂ OH
1914	1921	3385 A→G	K689R	PCR ^f
1915	1922	3384 A→G	K689E	PCR
2120	2122	3384 A→T 3385 A→G 3386 A→C	K689C	SDM
1916	1923	3399 A→C	T694P	PCR
1917	1924	3399 A→C 3683 A→G	T694P	PCR
2149	2156		K563C K689C	Cloning ^g
2108	2106	3302 T→C 3307 A→G 3320 T→A 3399 A→C	Y663C T694P	PCR
2233	2237	3315 A→G 3381 G→A	T666A G688R	PCR
2232	2236	3319 G→A 3375 T→C	R667H S686P	PCR
2231	2235	3381 G→A 3403 A→G	G688R Q695R	PCR
2107	2105	3406 A→G 3489 T→C	Y696C T724A	PCR
2230	2234	3404 A→G 3405 T→C 3451 A→G	Y696H E711G	PCR
<i>lktA</i>				
2181	2182	2804 A→T 2805 A→G 2806 A→C	K554C	SDM
2157	2159	2805 A→C 2806 A→C	K554T	SDM ^h
2187	2188	3196 C→G	N684K	
2183	2184		K554C N684K	Cloning ⁱ

^a Strain designation of *hlyA* in *trans* to *hlyCBD*.

^b *hlyA* base pair numbering from reference 8; *lktA* base pair numbering from reference 16.

^c SDM, site-directed mutagenesis as described in the text.

^d NH₂OH refers to random mutagenesis by hydroxylamine as described in the text.

^e This mutation was isolated twice independently.

^f PCR, PCR random mutagenesis as described in the text.

^g Restriction endonuclease fragments containing pWAM2119 and pWAM2120 mutations to make the double mutation.

^h Site-directed mutagenesis mutation originally described in reference 9.

ⁱ Mutagenesis as described in footnote *f* above but using plasmids pWAM2181 and pWAM2187.

TABLE 3. Lysis of sheep erythrocytes and BL-3 and Raji cells by different *E. coli* hemolysin and *P. haemolytica* leukotoxin mutants

HlyA or LktA form	% Lysis (mean $\pm$ SEM)			D12 MAb reactivity ^b
	sRBC ^a	BL-3 cell	Raji cell	
HlyA	90 $\pm$ 5	100 $\pm$ 0	60 $\pm$ 9	+
pro-HlyA	0	0	0	—
HlyA _{K563T}	0	45 $\pm$ 11	0	+
HlyA _{K563C}	21 $\pm$ 1	59 $\pm$ 11	2 $\pm$ 1	+
HlyA _{E684K}	92 $\pm$ 5	89 $\pm$ 9	53 $\pm$ 9	— ^c
HlyA _{G688R}	5 $\pm$ 2	11 $\pm$ 8	2 $\pm$ 1	—
HlyA _{G688E}	3 $\pm$ 1	14 $\pm$ 7	3 $\pm$ 3	—
HlyA _{G688K}	4 $\pm$ 2	11 $\pm$ 6	0	—
HlyA _{K689R}	11 $\pm$ 2	41 $\pm$ 13	0	—
HlyA _{K689E}	0	2 $\pm$ 1	2 $\pm$ 2	—
HlyA _{K689C}	0	2 $\pm$ 1	3 $\pm$ 3	—
HlyA _{K563C,K689C}	0	1 $\pm$ 1	0	NT
HlyA _{T694P}	0	44 $\pm$ 7	0	—
HlyA _{Y696F}	86 $\pm$ 2	86 $\pm$ 6	28 $\pm$ 4	+ ^c
HlyA _{Δ626-673}	0	0	0	+ ^c
HlyA _{Δ673-699}	0	0	0	— ^c
HlyA [*] _{ad}	85 $\pm$ 5	99 $\pm$ 0	54 $\pm$ 9	+
HlyA [*] _{K690M}	0	41 $\pm$ 8	2 $\pm$ 1	—
HlyA [*] _{Δ238-259}	0	2 $\pm$ 1	0	NT
HlyA [*] _{Δrepeat #5}	89 $\pm$ 4	60 $\pm$ 2	14 $\pm$ 6	NT
LktA	0	76 $\pm$ 4	0	—
LktA _{K554T}	0	49 $\pm$ 13	0	NT
LktA _{K554C}	0	45 $\pm$ 5	0	NT
LktA _{N684K}	0	79 $\pm$ 3	0	—
LktA _{K554C,N684K}	0	47 $\pm$ 4	0	NT

^a sRBC, sheep erythrocyte.^b +, positive for reactivity; —, negative for reactivity; NT, not tested.^c D12 reactivity reported previously (25); lytic assay reperfomed for the purpose of side-by-side comparison.^d HlyA^{*} is the allelic form of HlyA expressed from recombinant plasmids encoding the *hly* genes from pHly152 (18).

grounds with or without *hlyC* recombinant plasmids in *trans*. Cellular lysates from a fusion containing HlyA_{S608-T725} residues were tested for reactivity to anti-HlyA polyclonal antibodies and MABs. Shown in Fig. 1 are immunoblots of these fusions demonstrating the HlyC dependence for the D12 epitope by HlyC-independent reactivity to a second anti-HlyA MAB, D1, which has an epitope proximal to that of D12 in the HlyA_{S608-T725} sequence (25).

Stanley et al. reported that arginine and leucine substitutions at either of the two acylated lysines result in loss of *in vivo* hemolytic activity in culture supernatants (27). The different HlyA substitution mutants isolated in this study were initially scored for their hemolytic phenotype by growth on sheep erythrocyte agar plates. We observed that with the *hlyA* mutations studied in this laboratory, a mutant with an HlyA_{K689R} substitution homologous to that described above loses its D12 reactivity but retains a small discernible zone of beta-hemolysis surrounding colonies grown on blood plates. To examine this observation further, the effects of the new *hlyA* mutations on cytotoxic activity were studied in lytic assays against different cellular targets. Summarized in Table 3 are data demonstrating that the cytotoxicity against Raji cells is readily lost by substitutions at either of two targeted lysines alone but that the relative degree of loss of activity against either erythrocytes or BL-3 cells appears to depend on the amino acid substituted at the respective lysine positions. To better illustrate the relative changes in the phenotypic patterns of these mutants, Fig. 2 shows representative toxin doses versus cytotoxicity curves. Such analyses were performed to generate the percent cytotoxicity levels presented in Table 3, where the listed percent

cytotoxicity represents the level of cellular lysis at the highest toxin dose available. At present we are unable to acquire these mutant toxins at higher concentrations and still maintain cytotoxic activity. The percentages of cytotoxicity listed are intended to simplify the discussion for each mutant. The slopes and plateaus of the dose-response curves are more informative, because they reveal the relative changes in mutant toxin efficiency. These data suggest that the retention of a single acylation site results in a form of HlyA that has significant cytotoxicity. From Fig. 2A and B, the estimated toxin doses leading to 50% lysis for sheep erythrocytes and BL-3 cells are the same (0.2 ng). This indicates that although the two cell types have sensitivities similar to that of the wild-type hemolysin, the mutants with putatively only a single acylation can still lyse BL-3 cells but are more dramatically affected in their ability to lyse erythrocytes. The recombinant construction of the HlyA_{K563C,K689C} double substitution results in a mutant toxin which has lost cytotoxic activity against all three cell types examined. In cytotoxicity assays this mutant protein behaves similarly to the HlyC[−] pro-HlyA form. The cytotoxicity levels for mutant toxins with alterations outside of the two HlyA acylation sites (e.g., HlyA _{Δ 238-259} and HlyA _{Δ 626-673}) show that loss of cytotoxicity against one cell type is accompanied by a general loss in cytolytic activity.

The HlyA_{E684K}, three HlyA_{G688}, and HlyA_{T694P} substitutions each result in loss of D12 reactivity, but their cytotoxic activities are affected differently. For example, the HlyA_{E684K} variant possesses nearly wild-type levels of activity against all three cell types, whereas three different substitutions at HlyA_{G688} yielded mutant toxins with marginal lytic activity against the same cells. The lytic phenotype for HlyA_{T694P} mutant has no detectable erythrolytic or Raji cell cytotoxicity but retains almost 50% of the wild-type BL-3-lytic activity.

The normally nonhemolytic *P. haemolytica* leukotoxin is converted into a hemolytic toxin by substitution of the HlyA_{K563-H739} sequence for the homologous LktA_{K554-N739} region in LktA (9). This HlyA region includes the two acylated lysines. A search for lysines positioned at similar sequences in LktA indicates that the proximal lysine, LktA_{K554}, is present, but at the region encompassing the distal site, there is an LktA_{N684} equivalent to HlyA_{K689} (27). No information is available on what sites in LktA are acylated by LktC, although it is known that in an *E. coli* recombinant background, *hlyC* in *trans* to *lktA* will result in

FIG. 1. Immunoblots of cell lysates from different *E. coli* backgrounds containing GST-HlyA gene fusions. Two different GST-HlyA recombinant plasmids were introduced into four different *hly* backgrounds, listed at the tops of the blots. In the left four lanes of each immunoblot are lysates of cells containing a pGEX-2T GST-HlyA recombinant in which there is a +1 frameshift at the GST and HlyA_{S608-T725} junction site. The right four lanes of each blot have lysates from cells containing the gene fusion in frame. Control lanes contain the background strain harboring the vector plasmid pACYC184. The arrow indicates the immunoblot signal representing the appropriately sized fusion peptide for the in-frame gene fusions. The MAB probes used in each blot are listed at the bottom of the figure. The immunoblotting conditions and signal detection are similar to those described previously (25).

FIG. 2. Dose dependencies of different toxin mutants. Shown are data representative of an experiment in which the amounts of cellular lysis effected by different concentrations of a set of mutant toxins are compared. The conditions for the lysis assays for each of the cell types and the method for measuring toxin concentration are described in the text. (A) Levels of lysis of sheep erythrocytes as monitored by release of A_{570} material versus different toxins. OD 570, optical density at 570 nm. The key to the right indicates the identities of the different HlyA mutants examined in this experiment. Percent cytotoxicity of BL-3 cells (B) and Raji cells (C) versus the same group of mutants described for panel A. The three sets of lysis assays shown were performed on the same day with the same preparations of toxins.

active LktA leukotoxic activity with a target cell specificity similar to that of the wild-type leukotoxin. We hypothesized that a lysine substitution at LktA_{N684} would provide an additional site for LktA acylation and a subsequent change in its cytolytic capabilities. The cytotoxic activities of LktA_{N684K} produced in the *E. coli hylCBD* background were similar to those of the wild-type LktA in the same background (Table 3). Because HlyC acylates HlyA at two lysine positions at least under in vitro conditions (27) and LktA shares only one lysine comparable to those in HlyA, it was inferred that LktA is acylated once by HlyC. Two different substitutions, LktA_{K554T} and LktA_{K554C}, were isolated by site-directed mutagenesis to examine the effect of the loss of the putative LktA acylation site. The former mutant protein was preliminarily described in our earlier publication on hybrid toxin construction (9). The LktA_{K554T} substitution was described as a mutant toxin with low but detectable levels of BL-3-lytic activity (~1% of wild-type levels). This *lktA* mutation was subcloned into a new vector background (pBS+) to yield pWAM2157. This LktA mutant along with the new LktA_{K554C} mutant was used in lytic assays which have undergone some recent modifications that enhance their sensitivities (25). At similar doses the LktA_{K554T} and LktA_{K554C} mutants possess 49 and 45% of the wild-type levels of BL-3-lytic activity, respectively. A LktA_{K554C,N684K}

double mutant was constructed by subcloning and was tested in the lytic assays. The BL-3-lytic phenotype of this mutant is the same as that of the LktA_{K554C} variant.

The genetic analysis of the HlyA activation sites is well complemented by sequence alignments of these regions among the different RTX toxins. Shown in Fig. 3A and B are alignments of the available RTX toxin amino acid sequences around the lysines described above. The alignment corresponding to the distal HlyA site contains only 6 of the 12 toxin sequences because the residue corresponding to HlyA_{K689} in the other 6 toxin sequences is either an asparagine or a serine, which would not be acylated via the amide linkage by the HlyC apparatus (27). In the comparison of the primary sequences for the two sites (summarized in the Fig. 3C histograms), only glycines preceding the two lysines are strictly conserved. In these two RTX toxin regions, the conserved positions at one site indicated in the Fig. 3C histogram are not matched in their spacing relative to conserved amino acids for the glycine-lysine pair at the other site. Analysis of the predicted HlyA secondary structure by either the Chou-Fasman method (4) or that of Robson and coworkers (10, 24) indicates that the lysines occur at potential bends that may be bracketed by alpha-helices. When examined on the basis of helical wheel projections of the possible alpha-helices, the conserved, charged residues pre-

FIG. 3. Amino acid alignments of RTX toxin polypeptide sequences at the regions surrounding the HlyA_{K563} and HlyA_{K689} sites. The sequences of the different RTX toxin polypeptides surrounding the proximal HlyA_{K563} site (A) and the distal HlyA_{K689} site (B) are listed and numbered to the left of the panels. The sequence alignments were performed as previously described (30). (C) Histogram summaries for the amounts of conservation of amino acids for the alignments shown in panels A and B, respectively. *, position of the lysine in each toxin sequence that corresponds to the in vitro-acylated sites in HlyA (27).

ceeding the lysines at either site, although not identical at each site, appear to closely occupy the same longitudinal half of the respective alpha-helices. In the protoxin form, the two modified HlyA regions represent two of the four sequence spans that have the greatest likelihood of being surface exposed by the predicted method of Emini et al. (7). The most likely surface-exposed domain for the prohemolysin is the HlyA₅₀₀₋₅₂₀ region, which curiously also possesses glycine-lysine amino acid pairs in the RTX toxin alignments.

DISCUSSION

One of the major discoveries reported here is that *E. coli* hemolysin lytic activities against sheep erythrocytes and Raji cells depend more on the presence of both acylated lysines in HlyA than does its cytotoxic activity against BL-3 cells. The lytic activities of mutant toxins with substitutions at either the HlyA_{K563} or HlyA_{K689} positions are more adversely affected for sheep erythrocytes and Raji cells than for BL-3 cells. This is best exemplified by the HlyA_{K563T} and HlyA_{K689R} mutants, which in dose-response studies have little detectable lytic activity against sheep erythrocytes and Raji cells but which have at least 50% of the wild-type hemolysin BL-3 cytotoxicity levels. The amino acids substituted at these lysines also result in variant toxins with different degrees of loss of the lytic activities. For example, the erythrolytic activity of HlyA_{K563C} is less adversely affected than that of the HlyA_{K563T} mutant. This is also evident for the four substitutions at the HlyA_{K689} site. The HlyA_{K689R} mutant is least affected in its BL-3 cytotoxicity, whereas the HlyA_{K689E} and HlyA_{K689C} mutants are essentially inactive against BL-3 cells. The glycines preceding the acylated lysines are the only residues within the primary sequence of individual RTX toxins that are absolutely conserved in the amino acid sequence alignments presented in Fig. 3. There-

fore, we reasoned that substitutions at the HlyA_{G688} site would have alterations in cytolytic activities similar to those substitutions at the neighboring HlyA_{K689} position because they would interfere with the acylation reaction at that site. There are no data available here that indicate that the acylation at HlyA_{K689} is blocked by the HlyA_{G688} substitutions. The loss of the D12 MAb reactivity for these mutant proteins could be related to a glycine contribution to the antibody epitope alone. However, the uniform loss in lytic activities for the HlyA_{G688} mutants, compared with the unequal pattern of loss of lytic activities by the different HlyA_{K689} mutants which can no longer be acylated at position 689, suggests that a glycine residue proximal to the lysine position plays a structural role in the function of the hemolysin larger than just one of recognition by the acylation mechanism. Because the HlyA glycine-lysine pair at position 688 to 689 is predicted to be within a flexible loop immediately distal to a charged alpha-helix, we hypothesize that at the glycine position, free rotation of the polypeptide backbone can occur. This permits potential structural movement of this region of the protein, and such hypothetical movement could occur when the lysine becomes acylated or afterwards when the fatty acid-modified toxin interacts with host cell membranes.

Another important finding described here is the observation that the *P. haemolytica* leukotoxin substitution LktA_{K554C} retains 59% of the lytic activity of LktA activated and secreted in an *E. coli hlyCBD* background. The lysis data for the mutants do not directly address whether the wild-type LktA_{K554} site is acylated in this setting. However, the results do imply that LktA has an alternative acylation site(s) that is used by the HlyC-dependent activation process. The site(s) would have to be different from those identified for either the *E. coli* hemolysin or the *B. pertussis* adenylate cyclase/hemolysin. If a glycine-lysine pair represents the minimum for a consensus site of the RTX HlyC-dependent activation process, then there are nine additional glycine-lysine pairs in the predicted LktA sequence besides the G-553-K-554 pair. The LktA positions of the glycines in these pairs are 429*, 492*, 503*, 526, 668, 753, 772, 781, and 886. The asterisks indicate glycine positions of those pairs which align with similar pairs in HlyA and most other RTX toxins. These three sites also possess charged residues neighboring either side of the glycine-lysine pair and are predicted to be surface exposed regions of LktA. It will be interesting to see by direct biochemical methods where the native and recombinant LktA molecules are modified.

The observation by Hackett et al. (12) that acylation of the *B. pertussis* adenylate cyclase/hemolysin occurs differently in *B. pertussis* and *E. coli* backgrounds with different consequences for hemolytic activity led to our substitution of a lysine at the LktA_{N684} position. The hypothesis was that the availability of a lysine at this LktA site would permit acylation and a gain of erythrolytic function. There is no direct evidence presented here that the LktA_{N684K} mutant protein becomes acylated, but whether or not the proximal LktA_{K554} site is present, the LktA_{N684K} substitution does not have hemolytic activity in an *E. coli* HlyC background. The observation by Hackett et al. combined with the evidence discussed above for the acylation of LktA at a site other than LktA_{K554} raises the possibility that the alternative LktA acylation(s) could inhibit hemolytic activity as does the acylation of the *B. pertussis* adenylate cyclase/hemolysin at CyaA_{K680} in an *E. coli* background (12).

The results with the GST-HlyA gene fusions indicate that in an in vivo setting, a relatively small region of the entire HlyA polypeptide is needed for recognition by the HlyC activation mechanism. The data presented here show that the bulk of the Ca²⁺-binding repeats distal to the HlyA_{K689} site and the ex-

tracellular targeting sequence at the carboxy terminus are not necessary for this process. Additional deletion analysis of the HlyA_{S608-T725} region by the gene fusion approach could further localize the HlyA sequences needed for the modification, but perhaps the more important issues related to this are the structural and functional consequences of the fatty acids added to the HlyA protein. To study these in greater depth, the gene fusion described here represents a new reagent for prospective biochemical and biophysical studies.

In summary, these results continue to support the hypothesis that there are RTX toxin structures that confer different host and cell type-cytotoxic specificities. The activation of the RTX toxins by the RTX HlyC-like gene products appears to play an important role in this intriguing phenotype.

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