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Identification of RTX Toxin Target Cell Specificity Domains by Use of Hybrid Genes

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The *Escherichia coli* hemolysin (HlyA) and *Pasteurella haemolytica* leukotoxin (LktA) are cytolytic toxins encoded by genes belonging to the recently described RTX gene family. These cytotoxins are, respectively, 1,023 and 953 amino acids in length and are encoded by genes within identically organized operons. They share 45% amino acid sequence identities but differ in their target cell specificities. In vitro-derived recombinant hybrid genes between *hlyA* and *lktA* were constructed by using restriction endonuclease sites created by oligonucleotide site-directed mutagenesis. The cytolytic activity of hybrid proteins was investigated using as targets sheep erythrocytes and two cultured cell lines from different species (BL3, bovine leukemia-derived B lymphocytes; and Raji, human B-cell lymphoma cells). HlyA is cytolytic to all three cell types. LktA lyses only BL3 cells. Among the hybrid proteins displaying cytolytic activity, the striking finding is that the hemolytic activity of several LktA-HlyA hybrids was independent of any cytolytic activity against either cultured cell species. The hemolytic activity was associated with the HlyA region between amino acids 564 and 739. Structures that are critical for HlyA cytolytic activity against BL3 or Raji cells were destroyed when LktA-HlyA and HlyA-LktA hybrids were made, respectively, at amino acid positions 564 and 739 of HlyA. In contrast to HlyA, which lysed the two different cultured cell lines with equal efficiency, Lkt-HlyA hybrids possessing the amino-terminal 169 residues of LktA lysed BL3 cells more efficiently than Raji cells. This suggests that a significant but not exclusive element of the LktA ruminant cell specificity resides in the amino-terminal one-fifth of the protein. A molecular model of the functional domains of HlyA and LktA is presented.

The RTX gene family, coding for related cytolytic exotoxins produced by different gram-negative bacteria, has recently been described (for a review, see reference 50). The first and best characterized member of this family is the *Escherichia coli* hemolysin (HlyA). When the *Pasteurella haemolytica* leukotoxin (LktA) was cloned and its gene was sequenced (20, 30, 45, 46), it was discovered that HlyA and LktA share unambiguous homology of their deduced amino acid sequences. Since then, other related toxins have been described, including the *Actinobacillus actinomycetemcomitans* leukotoxin (AaLtA), the *Actinobacillus pleuropneumoniae* hemolysin (AppA), and the *Bordetella pertussis* adenylate cyclase/hemolysin (CyaA) (10, 17, 19, 25). The related toxins share a striking structural feature consisting of tandemly arranged repeats of a nine-amino-acid sequence (L/I/F-X-G-G-X-G-N/D-D-X). This common trait led to the name repeats in toxins (RTX) family (52).

These toxins are secreted into the culture medium by a unique, leader peptide-independent process (9, 11, 18, 30). Two genes, *hlyB* and *hlyD*, located downstream of the structural gene, encode proteins involved in the secretion of the toxins (12, 34, 46, 48). The C-terminal 1/10 of HlyA (approximately positions 900 through 1023) participates in the HlyB- and HlyD-dependent secretion process across the cell envelope (13, 18, 26, 33). Among the RTX exoproteins there is little amino acid sequence identity in this C-terminal region. However, when *hlyBD* are provided in *trans* to *lktA* or *hlyA* in an *E. coli* background, LktA is still secreted into the culture supernatant, although not as efficiently as HlyA (15, 21). Recently, a third gene, *tolC*, located outside of the *hly* determinant and encoding an outer membrane protein, has been shown to be required for secretion of HlyA into the medium (49). In the RTX operons, the structural *A* gene is always 3′ to a *C* gene whose product is necessary for production of active toxins (2, 15, 39, 40). The *C* gene-mediated modification of the A proteins remains unknown.

HlyA does have a wide range of target cells including not only erythrocytes from different species (mice, sheep, cows, horses, and humans) (5) but also different types of nucleated cells such as rat and human renal tubular cells (23, 38) and human leukocytes and bladder epithelial cells (7, 15, 16, 24). In contrast, the cytolytic activity of the *P. haemolytica* leukotoxin is limited to ruminant leukocytes or cultured cells of ruminant leukocyte origin (1, 15, 22, 44). The mechanism for the target cell specificity of these toxins remains unclear, but we previously showed that it is independent of the *C* gene-mediated modification process (15).

In the present study, we took advantage of the gene sequence sequence similarity between *E. coli hlyA* and *P. haemolytica lktA* to create reciprocal pairs of hybrid toxin genes. Our observations and tentative conclusions about the domains of LktA and HlyA responsible for their different target cell specificities are presented.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* DH1 [F^- *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44*] and *E. coli* JM101 (F′ *supE traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*) were used as hosts for recombinant plasmids and phages. Uracil-containing single-stranded DNA was obtained from *E. coli* CJ236 (*dut-1 ung-1*

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TABLE 1. Oligonucleotides used in in vitro site-directed mutagenesis experiments

Substrate and gene position (bp)[a]	5'-to-3' sequence[b]	Restriction site created
hlyA, 1848–1868	CGCTTTTGCTAGCTCAGAAGA	*Nhe*I
lktA, 1021–1041	AGCTTTAGCTAGCGCATGTTG	*Nhe*I
lktA, 1658–1681	AGGCAATCGGTGCACCAATAACCG	*Apa*LI
hlyA, 2995–3016	TATTCATAGGTACCGGACTGCCTCC	*Kpn*I
lktA, 2164–2188	ATTCATAGGTACCTGTTTGTACCGC	*Kpn*I
lktA, 2703–2727	ATCACCACCAtGGAAGGCATCATTG	*Nco*I

[a] Numbers refer to the sequences published by Felmlee et al. (12) for the *E. coli* hemolysin gene (*hlyA*) and by Lo et al. (30) for the *P. haemolytica* leukotoxin gene (*lktA*).

[b] Underlined bases represent substituted nucleotides.

thi-1 relA1) transformed with the appropriate recombinant M13 phages.

LB and YT media were used and supplemented with the appropriate antibiotics (ampicillin [100 μg/ml] and chloramphenicol [20 μg/ml] [Sigma, St. Louis, Mo.]) as previously described (15).

DNA manipulation and plasmid construction. Rapid plasmid DNA preparation was done by the method of Birnboim and Doly (3). Large-scale plasmid DNA purification was performed similarly except that the DNA was further purified by CsCl-ethidium bromide density gradient centrifugation (35). Recombinant DNA procedures involving restriction endonucleases and T4 DNA ligase were performed as described by Maniatis et al. (35). Bacteriophage particles were prepared essentially by the protocol accompanying the dideoxy-sequence analysis kit from New England Biolabs (Beverly, Mass.).

The vectors pUC18, pACYC184, and M13mp18 have been previously described (8, 37). pWAM581 (*hlyCABD* in pACYC184), pWAM826 (*hlyA* in pUC18), pWAM827 (*lktA* in pUC18), and pWAM956 (*lktA* in M13mp18) were described earlier (15, 51). pWAM826bis (*hlyA* in M13mp18) was constructed by insertion of a 3.6-kb *Sac*I-*Hin*dIII fragment from pWAM826 into *Sac*I-*Hin*dIII-digested M13mp18. An *hlyCBD* subclone in pACYC184 was created by in vitro deletion of the 1.9-kb *Tth*111I-*Mlu*I *hlyA* fragment from pWAM581.

Site-directed mutagenesis and mutant analysis. Mutations were created by using an oligonucleotide-directed in vitro mutagenesis procedure described by Kunkel (28). Uracil-containing single-stranded DNA from pWAM826bis (*hlyA*) or pWAM956 (*lktA*) was used as the substrate. Mutagenic oligonucleotides were synthesized on an Applied Biosystems 390A DNA synthesizer. The oligonucleotides are listed in Table 1. In vitro synthesis of the complementary strand was performed by using T7 DNA polymerase (Pharmacia), and the resulting phage DNA was transfected into *E. coli* JM101. Candidate mutants containing a newly created restriction endonuclease site were screened by digestion of the replicative form of phage DNA with the appropriate enzyme. Slot blot hybridization was also used for screening, in which candidate single-stranded phage DNA was blotted on nitrocellulose and hybridized with 10^6 cpm of the corresponding [γ-^{32}P]ATP-labeled mutagenic oligonucleotide per ml. Hybridization was carried out in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–10× Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin) at room temperature. Sequential washes of the filters at increasing temperatures was followed by autoradiography (Kodak X-Omat AR film). All putative phage mutations were confirmed by DNA sequencing by the dideoxynucleotide method (43), using the Sequenase kit (U.S. Biochemical).

A *Sac*I-*Hin*dIII DNA fragment containing the altered *A* gene sequence was transferred from M13mp18 into *Sac*I-*Hin*dIII-digested pUC18, with the *A* gene constructs placed under the control of the *lac* promoter. The phenotype of the mutants was checked by transcomplementation with pWAM974 (*hlyCBD*) in an *E. coli* DH1 background.

Hybrid construction. Plasmid DNA or phage DNA was digested at both the unique *Sac*I site of the multiple cloning site of pUC18 and the new unique site created by the mutagenesis procedure. Each fragment obtained was isolated by electroelution after electrophoresis on a 1% agarose gel. The appropriate fragments were then ligated to get reciprocal hybrid genes. WAM1039 (Lkt_{554}–$Hly_{564-739}$–LktA), which is a three-part hybrid, was constructed in a two-step process. A *Kpn*I restriction site was first created in the hybrid Hly_{739}–LktA at bp 3003 as described above. The *Sac*I-*Kpn*I fragment of this mutant was then replaced by the *Sac*I-*Kpn*I fragment from the *lktA Kpn*I mutant, resulting in the replacement of the HlyA N-terminal 563 amino acids by the LktA N-terminal 554 amino acids. The DNA sequences of all the hybrid junction sites were determined. The phenotype of the hybrids was determined by transcomplementation with pWAM974 (*hlyCBD*) into the *E. coli* DH1 background.

Immunoblotting. LB broth supplemented with the appropriate antibiotics was inoculated with a few colonies from an overnight culture plate and incubated at 37°C with shaking for 4 to 5 h. The bacterial cells were collected by centrifugation (8,000 × *g* for 30 s), and the supernatant was filtered (0.2-μm-pore-size Acrodisk; Gelman Sciences, Inc.). The supernatant proteins were precipitated with 10% trichloroacetic acid at 4°C for at least 1 h and collected by centrifugation (8,000 × *g*, 15 min, 4°C), and the pellets were resuspended in 1 M Tris (pH 9.0) and mixed with an equal volume of 2× crack buffer (0.13 M Tris [pH 6.8], 4% sodium dodecyl sulfate [SDS], 20% glycerol, 0.002 bromophenol blue, 2% 2-β-mercaptoethanol). The samples were subjected to discontinuous SDS-polyacrylamide gel electrophoresis by the method of Laemmli (29) with 10% polyacrylamide gels. Proteins were then transferred to nitrocellulose by the method of Towbin et al. (47). Following transfer, the nitrocellulose was blocked with 0.5% Tween 20 (Sigma) in phosphate-buffered saline. The proteins were probed independently with three different antibody preparations: a polyclonal antiserum against *P. haemolytica* LktA (provided by R. Y. C. Lo, University of Guelph) at a 1:500 dilution, a polyclonal antiserum against *E. coli* HlyA (our laboratory) at a 1:10,000 dilution, and an anti-HlyA monoclonal antibody, A10 (41), diluted 1:5,000. After a 90-min incubation, the blots were washed and the presence of bound antibodies was detected colorimetrically by the addition of either a goat anti-rabbit or anti-mouse immunoglobulin serum conjugated to alkaline phosphatase (Sigma).

Hemolysis assay. The hemolysis assay was performed as described previously (53) with the following modifications. Two hundred microliters of either cell-free supernatant prepared as described previously (15) or total bacterial cell culture was mixed with 10^9 sheep erythrocytes which had been washed and resuspended in saline plus 0.02 M $CaCl_2$ in a final volume of 1 ml. After an incubation of 30 min at 37°C, the unlysed erythrocytes were removed by centrifugation

(8,000 × *g* for 30 s) and the optical density at 540 nm was measured to determine the concentration of hemoglobin released into the supernatant. Results were expressed as the percentage of hemoglobin released compared with 100% release obtained with lysis of the erythrocytes by distilled water.

Cytotoxicity assay. Bovine leukemia-derived B lymphocytes (BL3 cells) and human B-cell lymphoma cells (Raji cells; ATCC CCL86) were provided, respectively, by G. Splitter and G. Byrne (University of Wisconsin). The cell lines were grown in suspension in RPMI 1640 (GIBCO) supplemented with L-glutamine (2 mM), gentamicin (50 mg/liter), streptomycin (50 mg/liter), and 10% equine (BL3 cells) or calf (Raji cells) serum (Hyclone) at 37°C with 5% CO_2. Target cells were washed twice and resuspended in RPMI 1640 to a concentration of 2×10^6 cells per ml. Fifty microcuries of sodium [^{51}Cr]chromate, (specific activity, 1 mCi/ml; Dupont, NEN Research Products) was added per ml of cell suspension, and the mixture was incubated at 37°C for 1 h on a rocking platform. After incubation, the cells were washed three times in RPMI 1640 to remove the unbound ^{51}Cr and adjusted to a concentration of 10^6 cells per ml. One-tenth milliliter of this suspension was mixed with 0.2 ml of either total bacterial cell culture or cell-free bacterial culture supernatant in a 96-well tissue culture plate (GIBCO) and incubated at 37°C in 5% CO_2 for 60 min. The plate was subjected to centrifugation (1,500 × *g* for 5 min), 0.1 ml of the supernatant containing the released ^{51}Cr was transferred to vials, and the radioactivity was counted in a gamma counter. The maximum and the spontaneous releases were defined as the counts per minute obtained when 1 N HCl and RPMI 1640, respectively, were added to the cells. The toxicity of the samples was related to ^{51}Cr release by the following formula: % release = [(mean test cpm − mean minimum cpm)/(mean maximum cpm − mean minimum cpm)] × 100.

RESULTS

HlyA and LktA mutant expression. The new unique restriction endonuclease sites created in *E. coli hlyA* and *P. haemolytica lktA* are shown in Table 2. In three cases, amino acid substitutions resulted from the generation of the necessary restriction endonuclease site. For the *Apa*LI site in *lktA*, an alanine was changed to a glycine at position 383 and the serine at position 384 was changed to an alanine. The other amino acid changes occurred with creation of the *Kpn*I sites for *hlyA* and *lktA*. In both cases a lysine (amino acid position 563 for HlyA and 554 for LktA) was replaced by a threonine. Trichloroacetic acid precipitates of cell-free, late-log-phase culture supernatants from all mutants were subjected to immunoblotting, using polyclonal antibodies against either HlyA or LktA. This showed that each of the mutants secreted a protein with the appropriate molecular size and in quantities similar to that of the wild type (110 kDa for HlyA and 102 kDa for LktA, data not shown). All mutants possessed hemolytic and leukotoxic activities similar to that of either native HlyA or LktA (data not shown), except the HlyA and LktA *Kpn*I mutants. During a 30-min incubation, the *Kpn*I HlyA mutant lysed only 2% of the sheep erythrocytes, compared with 72% for HlyA. The cytotoxic activities of both *Kpn*I mutants determined with BL3 cells were considerably decreased; i.e., the percentages of cytotoxicity for HlyA *Kpn*I and LktA *Kpn*I mutants were both just 1%, compared with 76 and 54% obtained with HlyA and LktA, respectively. Thus, substitution of one polar amino acid (lysine) for another polar but uncharged amino acid (threonine) resulted in almost complete loss of cytolytic properties for both of these toxins. The *Kpn*I mutants were still used to create hybrids between HlyA and LktA but with the recognition that the threonines would have to be replaced with lysines in the final hybrids for proper assessment of their toxic activities.

TABLE 2. Amino acid substitutions caused by generation of new restriction endonuclease sites

Toxin	Original[a]	New	Site
	GAA CTG GCA	GAG CTA GCA	*Nhe*I
HlyA	Glu-179–Leu–Ala	Glu–Leu–Ala	
LktA	GGT CTT GCT	GCG CTA GCT	*Nhe*I
	Ala-169–Leu–Ala	Ala–Leu–Ala	
		GGT GCA CCG	*Apa*LI
HlyA	—[b]	Gly-391–Ala–Pro	
LktA	GCT TCA CCG	GGT GCA CCG	*Apa*LI
	Ala-383–Ser–Pro	Gly–Ala–Pro	
	GGA AAA	GGT ACC	*Kpn*I
HlyA	Gly-562–Lys	Gly–Thr	
LktA	GGT AAA	GGT ACC	*Kpn*I
	Gly-553–Lys	Gly–Thr	
		TTC CAT GGC	*Nco*I
HlyA	—	Phe-739–His–Gly	
LktA	TTT AAC GGT	TTC CAT GGT	*Nco*I
	Phe-732–His–Gly	Phe–His–Gly	

[a] Underlines indicate the bases changed by mutagenesis.
[b] —, restriction site exists in the original.

Hybrid protein expression. Four pairs of reciprocal hybrids and a three-part hybrid were constructed between *hlyA* and *lktA* (Fig. 1). The hybrid genes were subcloned at the same position in pUC18 and expressed under the control of the *lac* promoter. When complemented in *trans* with *hlyCBD* (pWAM974), the cells containing each of these hybrids secreted a protein of the expected molecular size into the medium (Fig. 2). However, the amounts of hybrid protein secreted by the cells with the HlyA C-terminal part (WAM1171, WAM1127, WAM1043, and WAM1025) were slightly higher (two- to fourfold) than the amounts secreted by the cells with the LktA C-terminal part (WAM1170, WAM1161, WAM1046, WAM1022, and WAM1039). This coincides with the observation that more HlyA than LktA was secreted extracellularly by cells containing *hlyA* (WAM1015) than by cells containing *lktA* (pWAM1017) (Fig. 2) when they were transcomplemented with *hlyCBD*. This difference in hybrid protein amounts cannot be explained by the difference in LktA- versus HlyA-specific antibodies in the anti-LktA serum used because the same difference in extracellular antigen levels was observed when either an anti-HlyA polyclonal antibody or a monoclonal antibody (A10) raised against HlyA which cross-reacts with the glycine-rich repeats present in both LktA and HlyA was used (data not shown). This expression difference is more likely to be due to the relative inefficiency of the C-terminal LktA targeting sequence in interacting with the *E. coli* HlyB/D-TolC secretory apparatus.

Hemolytic activity of HlyA is associated with a 176-amino-acid portion of HlyA and is independent of its leukotoxic activity. Among the hybrids created, five showed hemolytic activity against sheep erythrocytes (WAM1171, WAM1127, WAM1043, WAM1022, and WAM1039) (Fig. 3). The hemo-

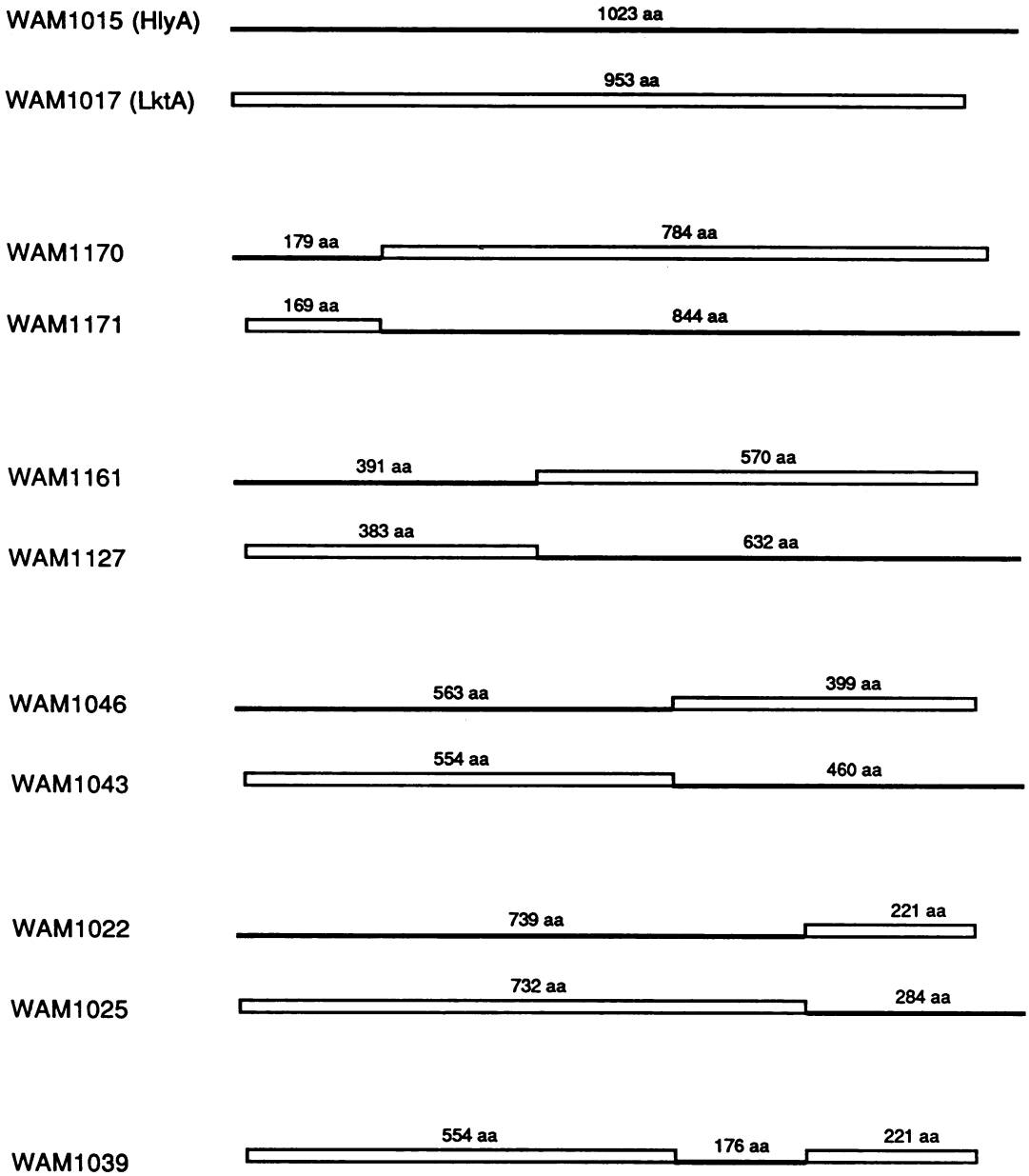

FIG. 1. HlyA and LktA hybrid proteins. The WAM1015 HlyA (thick line) and WAM1017 LktA (open bar) proteins are represented at the top. The numbers of amino acids (aa) in each portion of the hybrid proteins are given above each construct. The strain designation given on the left corresponds to the *E. coli* DH1 strain harboring each hybrid in *trans* to *hlyCBD* (pWAM974).

lytic activities in the supernatants of hybrids WAM1171 and WAM1127 were very unstable and were detected only when rapidly prepared samples from actively growing cultures were used. No hemolytic activity was detected with hybrids WAM1170, WAM1161, WAM1046, and WAM1025. However, hybrid WAM1022, which is similar to WAM1046 except that it contains an additional 176 amino acids from the HlyA N-terminal side, had hemolytic activity similar to that of HlyA. This suggests that the 176-amino-acid portion of HlyA between positions 564 and 739 is involved in the lysis of erythrocytes. This was confirmed by results obtained with hybrids WAM1171, WAM1127, WAM1043, and WAM1039. They all showed hemolytic activity, and their common portion of HlyA is this 176-amino-acid region. The hemolytic activities of hybrids WAM1043 and WAM1039 were in the same range when whole culture material was used (40% of lysis for hybrid WAM1043 and 30% for hybrid WAM1039) but were lower than those observed with hybrids WAM1171 and WAM1127 (90 and 96%, respectively). This suggests that the HlyA region upstream of the 176-amino-acid fragment, from positions 392 to 563, has an additional determinant facilitating HlyA hemolytic activity.

There was no significant difference in the hemolytic activities of whole bacterial culture material (cells plus culture supernatant) from hybrids WAM1043 (HlyA C terminus) and WAM1039 (LktA C terminus). It is curious that the supernatant material from hybrid WAM1039 had little hemolytic activity (4%) when compared with the supernatant material

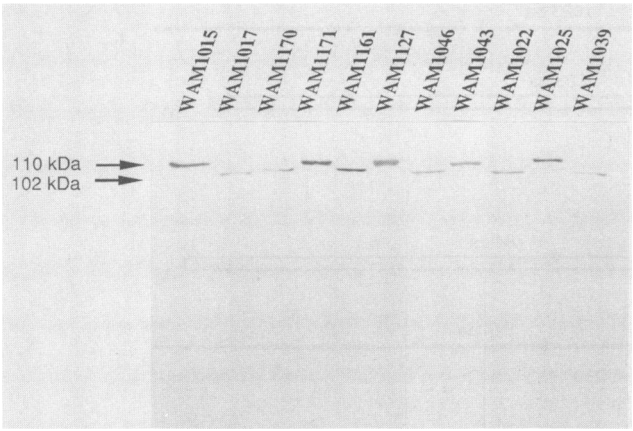

FIG. 2. Immunoblot of HlyA, LktA, and the hybrid proteins. Trichloroacetic acid-precipitated material from 1 ml of cell-free late-log-phase culture supernatant was subjected to electrophoresis on a 10% polyacrylamide–SDS gel and transferred to a nitrocellulose filter. The blot was probed with rabbit polyclonal anti-*P. haemolytica* LktA antibodies. The arrows at the left indicate the migration of HlyA (110 kDa) and LktA (102 kDa).

from hybrid WAM1043, which lysed 32% of the erythrocytes. This suggests that the HlyA C-terminal region from positions 739 to 1023 does not play a specific role in the lysis of erythrocytes but appears to provide an important stabilizing element for hemolytic activity of the cell-free material.

Culture supernatants containing HlyA lysed BL3 and Raji cells equally well (Fig. 4). Similar material produced by hybrids WAM1022 and WAM1039 showed hemolytic activity against sheep erythrocytes but surprisingly did not lyse BL3 or Raji cells (Fig. 4). The cytolytic activity of hybrid WAM1022 was investigated further, using as lytic targets human erythrocytes, another human B-cell lymphoma line (Daudi, ATTC CCL213), and human bladder epithelium cells

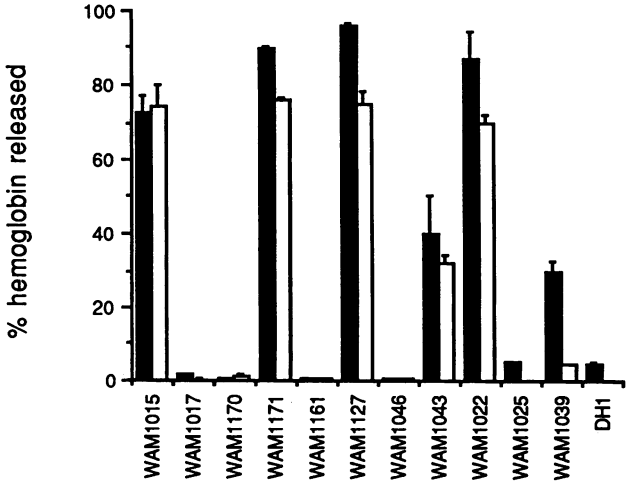

FIG. 3. Hemolytic activities of HlyA (WAM1015), LktA (WAM1017), and the hybrid proteins. The activities are expressed as the percentage of hemoglobin released after a 30-min incubation at 37°C with 10^9 sheep erythrocytes compared with 100% release obtained with lysis of erythrocytes with distilled water. The assays were performed with total late-log-phase cultures (solid bars) and cell-free supernatants (open bars). Error bars represent standard deviations. $n \geq 4$.

(T24 cells). Neither culture supernatant nor whole culture material from hybrid WAM1022 was cytolytic against the nucleated cells, but lytic activity was retained against the human erythrocytes (data not shown). Hybrid WAM1022 contains the first 739 residues of HlyA and the last 221 residues of LktA. The junction between the two toxins occurs within the third repeat such that the hybrid has the first two repeats of HlyA and the last eight of LktA. Hybrid WAM1039 has the identical junction point within the repeats.

The N-terminal region of LktA is implicated in target cell specificity. The construction of hybrids between HlyA and LktA also permitted investigation of which region(s) of LktA was responsible for its ruminant cell specificity. For this purpose we measured the cytolytic activity of each hybrid on both ruminant (BL3 and sheep erythrocytes) and human (Raji and erythrocytes) cells. We were disappointed to find that only two hybrids, WAM1171 and WAM1127, were cytolytic for BL3 cells, with 49 and 53% lytic activities, respectively (Fig. 4A). The same two hybrids also lysed Raji cells but with a much lower efficiency. Only 15 and 9% lysis was observed, respectively, for hybrids WAM1171 and WAM1127 (Fig. 4B). HlyA lysed BL3 cells (76%) and Raji cells (59%), whereas LktA lysed only BL3 cells (53%) and did not lyse Raji cells (<1%). Although hybrids WAM1171 and WAM1127 did not possess the same strict target cell specificity as LktA, they had greater cytolytic activities against BL3 cells than against Raji cells. The only portion of LktA common to WAM1171 and WAM1127 hybrids is the N-terminal 169 amino acids.

DISCUSSION

Because of the high sequence homology between the *E. coli* HlyA and *P. haemolytica* LktA cytolysins and their intriguing differences in target cell specificity (1, 6, 16, 22, 24, 44), we constructed hybrids of the toxin genes to better understand the function of the different toxin structural regions in the cytolytic process. To make reciprocal hybrid proteins with junctions that would be unlikely to cause local protein-folding alterations, restriction endonuclease sites were created in the genes corresponding to regions sharing polypeptide sequence identities based on an optimal sequence alignment (University of Wisconsin Genetics Computer Group gapfit program). In addition, identical toxin activation and transport functions were provided by transcomplementation with an *hlyCBD* subclone (pWAM974). It was previously shown that in an *E. coli* background, HlyC can activate LktA, which is then secreted into the medium by HlyBD (15). The determination of the cytolytic phenotype for each of the hybrid toxins by using different human and ruminant erythrocytes and cultured cells permitted identification of regions of HlyA and LktA that are in part responsible for their target cell specificities.

We encountered two difficulties with the hybrid gene approach for the study of RTX toxin target cell specificity. First, the quantification and normalization of the hybrid proteins by immunoblotting or enzyme-linked immunosorbent assay methods using polyclonal antibodies were problematic because of the various lengths of the antigens that were being tested. Monoclonal antibodies that are cross-reactive to HlyA and LktA helped with this problem. We have identified such monoclonal antibodies, and their epitopes reside in the glycine-rich repeat region (31). However, because HlyA and LktA do not share an identical number of repeats (13 versus 10), the number of epitopes for

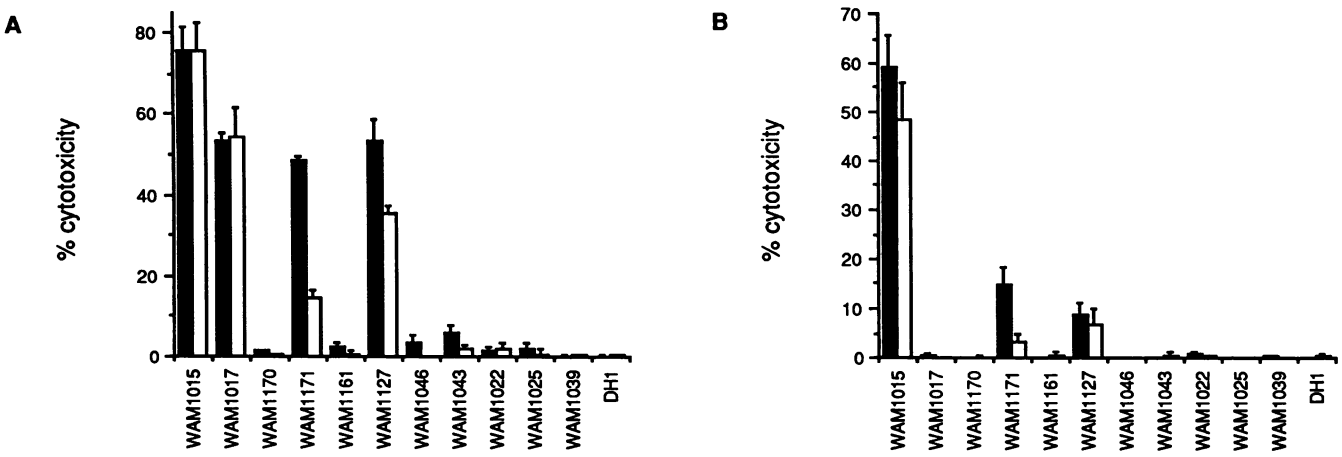

FIG. 4. Cytotoxic activities of HlyA (WAM1015), LktA (WAM1017), and the hybrid proteins against ^{51}Cr-labeled BL3 (A) and Raji (B) cells. The activities are expressed as the percentage of ^{51}Cr released after 1 h of incubation of total late-log-phase cultures (solid bars) or cell-free supernatants (open bars) with labeled cells (10^5 per assay). The maximum release and the spontaneous release were defined as the counts per minute obtained when 1 N HCl and medium, respectively, were added to the cells. Error bars represent standard deviations. $n \geq 4$.

these antibodies per toxin molecule is unknown and their use for quantification of the hybrids may still lead to equivocal normalizations of the toxins. To establish the range of linearity of the immunoblot signal to toxin concentration, serial dilutions of the different native and hybrid toxins were immunoblotted. The similarity in the decreasing strengths of immunoblot signals of our putatively normalized samples led us to conclude that errors in normalizing the concentrations of the hybrid toxins were unlikely to be greater than fourfold (data not shown). The second problem encountered was how to interpret the results for hybrids lacking any cytolytic activity. For example, the reciprocal hybrids of WAM1171 and WAM1127, WAM1170 and WAM1161, respectively, had little lytic activity against the target cells under the conditions used in this study (Fig. 3 and 4). The amount of hybrid protein produced and secreted by *E. coli* DH1 harboring hybrid WAM1170 or WAM1161 is less than what is obtained with hybrids WAM1171 and WAM1127. The hybrids with the LktA carboxy-terminal portion (including hybrids WAM1170 and WAM1161) are not secreted as efficiently as the hybrids with the HlyA carboxy terminus (Fig. 2). This expression difference is not due to plasmid copy number or transcriptional effects because all the hybrid genes are subcloned in the same orientation and into the same site of pUC18. The most likely explanation is that the putative interaction of the RTX A protein carboxy terminus with the HlyBD and TolC secretion machinery is less efficient for LktA than it is for HlyA. We do not believe that the expression difference and the possible normalization problem can account for the noncytotoxic activity of some of the hybrids. This is based on the observation that the WAM1022 and WAM1039 hybrids and LktA itself are less efficiently secreted than HlyA or any HlyA carboxy-terminal hybrid derivative, yet they still exhibited unambiguous cytolytic effects. We have performed a dose-response analysis of HlyA, LktA, and WAM1171 supernatants and whole culture material against BL-3 and Raji cells. These experiments indicate that under the assay conditions employed in this study there is not a linear relationship between toxin concentration and percentage of target cell lysis. Assays performed with eightfold greater toxin concentrations than those used in our standard protocol result in only 5 to 10% increases in target cell lysis (42). Therefore, despite our difficulty in normalizing the toxin concentrations, the inability of some hybrids to lyse cells is unlikely to be the result of inadequate concentrations of hybrid proteins in our assay. The identification of the functional defect in the inactive hybrids is under study. We do know that inactive hybrids are still capable of forming pores in artificial lipid bilayers (36). This suggests that their functional defect probably involves a structure necessary for an event prior to ion channel formation.

Perhaps the most surprising observation involved the hybrids which retained potent hemolytic activity but which had lost leukotoxic activity. For example, WAM1022, containing the amino-terminal 739 amino acids of HlyA and the carboxy-terminal 221 amino acids of LktA lysed sheep and human erythrocytes but did not affect either of the leukocyte-derived cultured cell lines (Fig. 3 and 4; 14). The activity of this hybrid toxin was also tested against Daudi (human B-lymphoma) and T24 (human bladder epithelium) cells, but neither were lysed (14). Because both LktA and HlyA normally have cytolytic activity against nucleated cells, the lack of cytolytic activity against either cell line by hybrid WAM1022 is probably due to a conformational irregularity that either disrupts or masks a critical structure, such as the ligand for a possible nucleated cell receptor or a membrane insertional domain which is different from that needed for hemolysis. The junction site of the WAM1022 hybrid occurs inside the glycine-rich repeat region such that the first two repeats of HlyA are joined to the last eight repeats of LktA. Because the first two repeats of HlyA are not completely identical to the corresponding repeats of LktA, they may not interact properly with the last eight repeats of LktA. An alternative hypothesis is that a particular tertiary structure cannot be achieved that involves parts of the hybrid structure outside of the repeats. This structure is crucial for leukotoxicity but is dispensible for hemolysis. Nonetheless, it remains fascinating that this hybrid protein still efficiently lyses sheep or human erythrocytes. A similar phenotype is known for an HlyA mutant in which a *Xba*I linker was inserted into *hlyA* at the unique *Bgl*II site corresponding to the region between the 11th and the 12th copies of the repeats. This in-frame insertion mutant still has 50% of

the wild-type HlyA hemolytic activity but does not lyse BL3 or Raji cells (42). Therefore, it seems that a specific structural feature of the repeat region is required for HlyA cytolytic activity against nucleated cells but this structure only marginally affects hemolytic activity.

Ludwig et al. found that HlyA mutants lacking three copies of the glycine-rich repeats require elevated amounts of calcium (100 mM) in the assay mixture in order to produce hemolytic activity (32). An interesting adjunct to the WAM1022 phenotype is that this hybrid has three less repeats than native HlyA yet behaves as an efficient hemolysin without a requirement for calcium concentrations greater than 10 mM in the liquid hemolysis assay mixture. The reason for this discrepancy is unknown, but the specific position of the deleted repeats may play an important role in causing the apparent calcium concentration dependency for hemolytic activity.

By comparing the phenotypes of hybrids WAM1171, WAM1127, WAM1043, WAM1022, and WAM1039, it appears that 176 residues of HlyA (positions 564 to 739) located upstream of the repeat region are sufficient to confer hemolytic activity to the normally nonhemolytic LktA when they are substituted for the corresponding region of LktA. This HlyA/LktA region shares 41% amino acid sequence identity, compared with 46% over the entire lengths of the two proteins. In order to achieve the optimal HlyA/LktA sequence alignment in this region, it is necessary to introduce two single and one double amino acid gap in LktA and one six-residue HlyA gap. The significance of these differences is unknown. Why the RTX leukotoxins do not lyse erythrocytes remains unclear, but our work indicates that appropriate amino acid substitutions in a discrete linear region of LktA can result in a hemolytically active protein.

It is important to point out that the WAM1043 hybrid, with a junction point at $LktA_{554}$–$HlyA_{563}$, has the identical hemolytic but nonleukotoxic phenotype of WAM1022. Therefore, we conclude that there are additional regions of HlyA and LktA which are critical for leukotoxic but not hemolytic activity. The structure of the $LktA_{554}$–$HlyA_{563}$ region is superficially similar to the glycine-rich repeat domain. Different algorithms which predict protein structure indicate that this area in both toxins is likely to be surface exposed and rich in β turns (54). It appears that assays of leukocyte lysis are a very sensitive means of assessing the functional significance of structural alterations in the cytolytic members of the RTX family.

There is indirect evidence indicating that the amino-terminal 169 amino acids of LktA are involved in its ruminant target cell specificity. Two hybrids, WAM1171 and WAM1127, which have in common the first 169 amino acids of LktA, were both more efficient at killing BL3 cells than Raji cells (Fig. 4). This phenotype is similar although not quantitatively identical to the way in which LktA lysed BL3 but not Raji cells. Albeit there is detectable lysis of human-derived nucleated cells by these hybrids, their pattern of lytic activity is different from that of HlyA, which lysed Raji cells as well as BL3 cells, whereas LktA did not lyse Raji cells under any of our experimental conditions. Because WAM1171 still lysed Raji cells (15% lysis, Fig. 4B), it suggests that some element of the HlyA specificity determinant for Raji cells is outside of its N-terminal 179-amino-acid region. These relative differences in cytolytic activities against nucleated cell targets shown by single RTX hybrid derivatives under identical assay conditions suggest that the target cell RTX toxin species specificity is a property different from the nucleated cell-versus-erythrocyte specificity previously discussed.

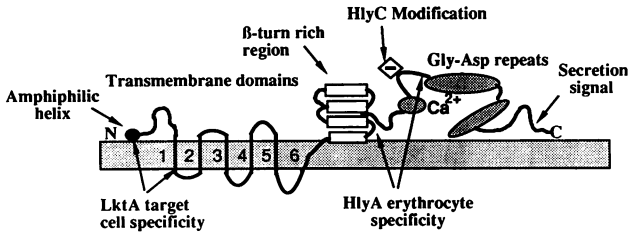

FIG. 5. Molecular model for interaction of HlyA and LktA with target cell membranes. The model assumes that the amino-terminal part of LktA initiates its interaction with a ruminant leukocyte cell surface. The region following this has lengthy runs of hydrophobic amino acids in both toxins. This region probably becomes integrated into the target cell membrane (as shown) or is an interior domain for a globular portion of the toxins. This is followed by a β-turn-rich region that is divided into two parts. From approximately position 500 to the beginning of the glycine-rich repeats at position 721 (HlyA) is one part, and the repeat region is the second part. For HlyA, the region between positions 563 and 739 contains a structure responsible for erythrocyte lysis. The location of the HlyC activation-specific epitope of the D12 monoclonal antibody resides in this region (41). The extracellular targeting signal for secretion of HlyA across the *E. coli* cell envelope resides in the C terminus (18, 26).

The sequence alignment of the N-terminal 169 amino acids of LktA and 179 amino acids of HlyA reveals several differences which may account for the divergent host cell specificities. First, there is little sequence identity among the RTX toxins within their first 50 residues (27). Second, in a comparison of HlyA and LktA sequences, two gaps are needed to achieve the optimal alignment. One occurs at residue 58 of HlyA and the other at LktA residue 164. Third, the predicted pI (9.2) for the N-terminal 200 amino acids of HlyA is basic. This is a trait common to all the RTX toxins with the exception of LktA, for which the pI for the region is 6.1. The significance of any of these structural or chemical differences between HlyA and LktA or other RTX toxins is unknown, but they represent potential features which could be assessed for their contribution to the putative target cell species specificity element of RTX leukotoxins.

Figure 5 represents a model for interaction of HlyA or LktA with the membrane of a target cell. The amino-terminal part of LktA is putatively involved in its ruminant cell specificity. It remains to be demonstrated that this is due to the presence of a receptor-binding domain involved in ruminant cell recognition. Following this region (residues 92 to 410 of HlyA and 89 to 392 of LktA) there are several successive stretches of hydrophobic amino acids which are likely toxin domains that span host cell membranes. The next sequence of 300 amino acids is probably rich in β turns and likely to harbor the HlyC modification site (41). In the case of HlyA, this region contains a structure which facilitates lysis of erythrocytes. The calcium-bound repeat region beginning at HlyA position 721 is needed to lyse erythrocytes as well as nucleated cells (4). An additional structure within the repeat region of HlyA is very critical for cytolysis of nucleated cells but not erythrocytes. Lastly, a poorly defined secondary or tertiary structure in the carboxy terminus is needed for HlyB-, HlyD-, and TolC-mediated export. Further experiments, including target cell binding assays, construction of hybrids among other members of the RTX family, and the isolation of specific mutations are needed to test the validity of different features of this model.

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ADDENDUM IN PROOF

The recent discovery that the HlyC activation of HlyA involves fatty acylation [J.-P. Issartel, V. Koronakis, and C. Hughes, Nature (London) **351**:759–761, 1991] suggests to us that our model of RTX toxin interaction with host membranes (Fig. 5) should be altered to show the HlyC-modified domain of HlyA closely associated with the host membrane.

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