Prelytic and Lytic Conformations of Erythrocyte-Associated

Escherichia coli Hemolysin

MAHTAB MOAYERI AND RODNEY A. WELCH*

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

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Flow cytometry was developed as a method to assess the conformation of erythocyte-bound Escherichia coli hemolysin polypeptide (HlyA). Topology of membrane-associated hemolysin (HlyA<sup>E</sup>) was investigated by testing surface accessibility of HlyA regions in lytic and nonlytic bound states, using a panel of 12 anti-HlyA monoclonal antibodies (MAbs). Hemolysin associates nonlytically with erythrocytes at 0 to 2°C. To test the hypothesis that the nonlytic HlyA<sup>E</sup> conformation at 0 to 2°C differs from the lytic conformation at 23°C, MAb epitope reactivity profiles at the two temperatures were compared by flow cytometry. Four MAb-reactive epitopes have distinctly increased reactivity at 0 to 2°C compared to 23°C. HlyA requires HlyC-dependent acylation at lysine residues 563 and 689 for lytic function. Toxin with cysteine substitution mutations at each lysine (HlyAK563C and HlyAK689C) as well as the nonacylated form of hemolysin made in a HlyC-deficient strain were examined by flow cytometry at 0 to 2 and 23°C. The three mutants bind erythrocytes at wild-type toxin levels, but there are conformational changes reflected by altered MAb epitope accessibility for six of the MAb-reactive epitopes. To test further the surface accessibility of regions in the vicinity of MAb-reactive epitopes, HlyAE was proteolytically treated prior to testing for MAb reactivity. Differences in protease susceptibility at 0 to 2° and 23°C for the reactivities of three of the MAb-reactive epitopes further support the model of two distinct conformations of cell-associated toxin.

MATERIALS AND METHODS

Bacterial strains and toxin preparation. WAM582(pSF<sub>4000</sub>) is a plasmid recombinant strain (DH10B with hlyC<sup>AC184</sup>) which has previously been described (25). WAM1824 is the source for wild-type toxin in most flow cytometry experiments. This strain is pWAM582(pSF<sub>4000</sub>) in the CL633 background (WAM1808) (5). The CL633<sup>+</sup> E. coli K-12 background strain results in approximately 20-fold-higher levels of HlyA production if hlyA is expressed from the complete hly operon compared to WAM582 expression. Cysteine substitution mutants were constructed by oligonucleotide-directed mutagenesis using the procedure of Kunkel (10). Mutations were subcloned into the hly<sup>CABD</sup> operon as previously described (25). WAM6935 is a Hly C<sup>−</sup> pro-HlyA construct of WAM582 and has been previously described (21). Bacteria were grown at 37°C in Luria-Bertani broth containing 20 µg of chloramphenicol per ml to an optical density at 600 nm of 0.75 to 0.85. Bacteria were pelleted, and culture supernatants passed through a 0.45-µm-pore-size Acrodisc were used as source of toxin.

Antibodies. Antibodies and their neutralization profiles have been initially described elsewhere (19). Epitopes for monoclonal antibodies (MAbs) G8, B7, and H10 have been further mapped by using a SPOTs kit (Genosys Technologies, The Woodlands, Tex.) (20). Epitopes for MAb G9, G3, and D12 were further mapped by mutational analysis (20). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG) and anti-rabbit IgG were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Flow cytometry experiments. Sheep erythrocytes (sRBC) used as targets in all experiments were obtained fresh and defibrinated with glass beads. Toxin was assayed with target cells by mixing 500 µl of a 0.5% (2.5 x 10<sup>7</sup> cells) sRBC suspension with 500 µl of toxin-containing culture supernatant and incubating the mixture for 30 min (room temperature) or 1 h (0 to 2°C), with occasional mixing. To prevent lysis in experiments performed at 23°C, all sRBC suspensions were made in dextran 4 buffer (30 mM dextran 4 [Serva Chemicals, Heidelberg, Germany] in 145 mM NaCl–45 mM KCl), and all washes were also performed in
this buffer. Dextran 4 buffer prevents hemolysin-induced lysis of erythrocytes by osmotic protection (3, 17). Erythrocytes with bound hemolysin (E HlyA) were washed twice to remove unbound toxin and resuspended in 100 ml of dextran buffer. Anti-HlyA monoclonal or polyclonal primary antibodies were added (dilutions are given in Fig. 1B) and incubated for 1 h. Erythrocytes were washed twice with 500 ml of buffer to remove unbound primary antibody, resuspended in 100 ml of 1:25-diluted FITC-conjugated goat anti-mouse or anti-rabbit IgG (Sigma), and incubated in the dark for 30 to 45 min. Cells were washed once in buffer and resuspended in 300 ml of dextran buffer or cold saline until the time of analysis. Erythrocytes were assessed for fluorescein-specific fluorescence with a FACStar flow cytometer (Becton Dickinson, Mountain View, Calif.), and 5,000 or 10,000 events were collected per sample. Mathematical analysis of data was performed by using the PCLYSIS flow cytometry analysis software program (Becton Dickinson). The negative controls in these experiments were EHlyA; E HlyA plus primary antibody; E HlyA plus secondary (FITC-conjugated) antibody; and E HlyA plus two irrelevant primary antibodies plus secondary (FITC-conjugated) antibody.

**RESULTS**

Detection of hemolysin bound to erythrocytes by flow cytometry. The first goal in these studies was to establish if flow cytometry could be used as a method to measure toxin binding to target cells. Polyclonal anti-HlyA antibodies were used to detect binding of wild-type hemolysin to dextran 4-protected erythrocytes at ambient temperature. Eighty to 90% of all E HlyA bind polyclonal antibody. Similar experiments were performed with nonacylated hemolysin and with cysteine substitution mutants (HlyA K563C and HlyA K689C). HlyA K563C has a 300-fold decrease in hemolytic activity, while non-acylated HlyA and HlyA K689C have no hemolytic activity (18). All three mutant toxins bind erythrocytes at both 0 to 2 and 23°C, as indicated by polyclonal binding. Figure 2 shows a representa-

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**FIG. 1.** (A) Schematic of HlyA showing structural regions and MAb epitopes. Designations and locations of MAb epitopes are shown above the horizontal bar. The thickest straight black region indicates the putative transmembrane domain. The open dotted region represents the calcium-binding repeats of HlyA, interrupted by the modification region containing acylation site lysine 689 and the D12 acylation-dependent epitope. Acylation sites are shown with asterisks at residues 563 and 689. (B) Epitope regions and concentrations of MAbs and the maximum HlyA detection titers by ELISA and immunoblotting (19, 21). FC titer is the dilution used in the primary antibody binding step of flow cytometry experiments.

**FIG. 2.** Wild-type toxin and nonacylated, pro-HlyA bind erythrocytes. Results of a representative experiment show the binding of equivalent amounts of toxin from WAM783 (Hly C - pro-HlyA) and WAM582 (wild type [WT]). Anti-hemolysin polyclonal antibody (1:1,000) was used to detect binding. The negative control is toxin-bound erythrocytes plus secondary FITC-conjugated antibody. All other negative controls (described in Materials and Methods) showed lower levels of fluorescence than this control. For each sample, 10,000 collected cells were assessed by flow cytometry.
tive experiment depicting the binding of wild-type hemolysin and nonacylated toxin to erythrocytes.

**MAb epitope accessibility and HlyA<sup>E</sup> topology.** To test the hypothesis that certain regions of HlyA<sup>E</sup> are membrane inserted while other regions are surface exposed, a panel of well-defined MAbs with epitopes throughout the toxin was used to probe the accessibility of different HlyA domains in toxin bound to erythrocytes (Fig. 1). These antibodies react with HlyA in its native soluble conformation, as indicated by enzyme-linked immunosorbent assay (ELISA) and native dot blots, as well as to its putatively denatured state in immunoblotting (19, 21) (Fig. 1). E<sup>HlyA</sup><sub>AE</sub> protected from lysis by dextran 4 buffer was tested for reactivity with each MAb at 23°C. For experiments performed with wild-type toxin, n = 19 at 23°C and n = 5 at 0 to 2°C; for all other experiments, n = 2 to 4. –, <10% of E<sup>HlyA</sup><sub>AE</sub> show fluorescence levels greater than that of negative controls; ND, experiment not done; I, inconclusive.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Wild type</th>
<th>K689C</th>
<th>K563C</th>
<th>Hly&lt;sup&gt;C&lt;/sup&gt;&lt;sup&gt;AE&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>23°C 0–2°C</td>
<td>23°C 0–2°C</td>
<td>23°C 0–2°C</td>
<td>23°C 0–2°C</td>
</tr>
<tr>
<td>G8</td>
<td>70 ± 4</td>
<td>17 ± 10</td>
<td>7 ± 4</td>
<td>5 ± 4</td>
</tr>
<tr>
<td>C10</td>
<td>52 ± 5</td>
<td>44 ± 10</td>
<td>4 ± 4</td>
<td>8 ± 4</td>
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<tr>
<td>E2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>H10</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B7</td>
<td>–</td>
<td>54 ± 9</td>
<td>1 ± 4</td>
<td>7 ± 4</td>
</tr>
<tr>
<td>B9</td>
<td>80 ± 3</td>
<td>82 ± 2</td>
<td>75 ± 4</td>
<td>5 ± 9</td>
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<td>55 ± 5</td>
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<td>D12</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>A10</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>B10</td>
<td>76 ± 11</td>
<td>18 ± 14</td>
<td>46 ± 5</td>
<td>28 ± 11</td>
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<tr>
<td>C7</td>
<td>15 ± 3</td>
<td>22 ± 1</td>
<td>1 ± 4</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>Polyclonal</td>
<td>88 ± 2</td>
<td>85 ± 4</td>
<td>85 ± 5</td>
<td>54 ± 5</td>
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</table>

* Values represent the percentages of erythrocytes with fluorescence greater than that of negative controls. Negative controls are described in Materials and Methods.

**MAb reactivity profiles for HlyA<sub>K689C</sub>, HlyA<sub>K563C</sub>, and nonacylated pro-HlyA.** The acylation-defective HlyA mutants were tested in flow cytometry experiments at 0 to 2 and 23°C (Table 1; Fig. 3). Similar to results seen with wild-type toxin, MAbs A10, D12, E2, and H10 show no reactivity with the mutant toxins bound to erythrocytes at either tested temperature (Fig. 3A). MAb D1 is the only antibody that shows consistent positive reactivity with all three erythrocyte-bound mutants at both temperatures (Fig. 3B). In contrast to results with wild-type toxin, a small percentage of cells bound with HlyA<sub>K563C</sub> showed low reactivity with MAbs B7, B10, and C7 at 23°C (Fig. 3B). Additionally, a significantly lower percentage of erythrocytes with HlyA<sub>K563C</sub> bound react with MAb G8, and this reactivity is lost at 0 to 2°C. HlyA<sub>K689C</sub> shows the same lowered reactivity with MAb G8 at both temperatures. All other reactivities for this mutant at 23°C indicate a reactivity profile similar to that for wild-type toxin, but with only 40 to 50% of the E<sup>HlyA</sup><sub>AE</sub> population having antibody-reactive sites on their surface, in contrast to the 70 to 90% seen with wild-type toxin (Table 1). Interestingly, HlyA<sub>K689C</sub> does not increase in the number of accessible MAb epitopes at 0 to 2°C. Its overall reactivity profiles are similar at the two temperatures (Table 1). Nonacylated pro-HlyA also shows altered MAb reactivities (Table 1). The striking result with this particular mutant is the lowered reactivity seen for MAb B9. It is clear from these observations that the mutant toxins bind erythrocytes with conformations different from that of the wild type at both 0 to 2 and 23°C.

**Susceptibility of accessible surface-exposed epitopes of HlyA<sup>E</sup> to proteolytic treatment.** To further test the surface exposure of positive-reacting MAb epitope regions and to investigate local conformational structure in the vicinity of these epitopes, HlyA<sup>E</sup> was treated with proteases prior to assessing MAb reactivity profiles. The reactivities for three of five reactive MAbs (B9, C7, and D1) are completely eliminated from
more than 50% of all E^HlyA^ at 23°C (Table 2; Fig. 4B). At 0 to 2°C, the reactivities of five of eight of the normally positive MAbs (B7, B10, C10, D1, and G8) are abrogated by proteolytic treatment (Table 2). The remaining 10 to 35% of proteolytically treated E^HlyA^ which retain reactivity with these MAbs show a 10- to 500-fold reduction in fluorescence intensity. This is a measure of the number of reactive epitopes per cell. The readily reactive epitopes for MAbs B9 and G3 are not susceptible to protease treatment at 0 to 2°C but are easily removed by proteolysis at 23°C. The G3 epitope is also relatively resistant to protease treatment at 23°C (Fig. 4A).

The N-terminal epitope region for MAb G8 is protease sensitive in the nonlytic conformation at 0 to 2°C (Table 2). In the lytic conformation, its MAb reactivity with proteolytically treated E^HlyA^ is reduced to 10-fold in terms of fluorescence intensity. Although this result indicates that a lower number of MAb-reactive sites exist per erythrocyte, all E^HlyA^ cells maintain toxin molecules with accessible MAB G8-reactive epitopes and the overall percentage of positive-reacting cells is unchanged (Fig. 4C). In this context, it is important to emphasize that the values in Table 2 represent the percentages of E^HlyA^ for which proteolytic treatment results in complete loss of a particular epitope; reductions in fluorescence intensity that do not affect the percentage of reactive cells are not included.

### Table 2. Loss of epitope regions and MAb reactivity with proteolytic treatment

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Avg % sRBC with MAb epitope removed by proteolysis ± SD^a^</th>
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<tbody>
<tr>
<td></td>
<td>23°C</td>
</tr>
<tr>
<td>G8</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>C10</td>
<td>NA</td>
</tr>
<tr>
<td>E2</td>
<td>NA</td>
</tr>
<tr>
<td>H10</td>
<td>NA</td>
</tr>
<tr>
<td>B7</td>
<td>NA</td>
</tr>
<tr>
<td>B9</td>
<td>66 ± 10</td>
</tr>
<tr>
<td>D1</td>
<td>80 ± 15</td>
</tr>
<tr>
<td>G3</td>
<td>18 ± 14</td>
</tr>
<tr>
<td>D12</td>
<td>NA</td>
</tr>
<tr>
<td>A10</td>
<td>NA</td>
</tr>
<tr>
<td>B10</td>
<td>NA</td>
</tr>
<tr>
<td>C7</td>
<td>100 ± 0</td>
</tr>
</tbody>
</table>

^a^ Average percentage of erythrocytes showing loss of MAb reactivity (fluorescence) when HlyA^ is treated proteolytically, n = 3 for all experiments except for MAbs G3, G8, and B9 (n = 4 to 6). NA, not applicable (MAb is nonreactive for HlyA^ with or without proteolytic treatment); I, inconclusive due to inconsistent results.
erythrocyte complex can be shifted from a prelytic state to a lytic one where additional MAb epitopes become unavailable for binding. Toxin association with erythrocytes at 0 to 2°C is characterized as a prelytic state, because following a shift to 23°C there is a lag period before lysis begins. This situation is in contrast to the immediate lysis that occurs at 23°C, when an osmotic protectant such as dextran 4 is removed from an E\textsuperscript{HlyA}\textsuperscript{p} suspension. Thus, the lytic structure of HlyA\textsuperscript{p} in these studies represents a functionally mature complex achieved at 23°C in the presence of a protectant such as dextran 4. We postulate that the prelytic and lytic states of hemolysin, as well as association of toxin with erythrocytes under nonlytic conditions (calcium-free conditions; nonacylated or mutant toxins) represent independent membrane-bound HlyA conformations. In this study, flow cytometry was used to study conformations of prelytic and lytic wild-type HlyA\textsuperscript{p} as well as the erythrocyte-membrane associated nonacylated toxin and two HlyA acylation mutants.

In the lytic state, at 23°C, MAbs tested for reactivity with E\textsuperscript{HlyA}\textsuperscript{p} show distinct positive and negative reactivities in a manner completely independent of and often in contrast to their immunoblot, native dot blot, and ELISA HlyA titers (Fig. 1). Thus, the flow cytometry results reported here do not reflect differences in the titer of MAb used to detect either the native or denatured soluble form of HlyA. Of the 12 MAbs that react with soluble HlyA, only 4 react with HlyA\textsuperscript{p} in the lytic state at 23°C. The MAb reactivity profiles indicate surface accessibility of the HlyA N terminus and the region between residues 594 and 640 (HlyA\textsubscript{594-640}). MAb G8 reacts with HlyA\textsuperscript{p}, and its epitope lies within the first 12 residues of HlyA. MAbs B9, D1, and G3 also react, and their epitopes exist within HlyA\textsubscript{594-640}. The eight nonreactive MAbs indicate that regions directly N terminal and C terminal to residues 594 to 640 are inaccessible for MAb reactivity. Domains which contain nonreactive MAb epitopes include regions on either side of the putative transmembrane domain and just N-terminal to the 563 acylation site, the 689 acylation site region, and the repeat region. The lack of reaction with epitopes within these domains could reflect membrane insertion and lack of surface accessibility for the associated regions. Alternatively, it is also possible that local protein folding in the vicinity of the tested epitope prevents MAb accessibility. Unfortunately, at present there are no MAbs available for the putative transmembrane region or the C-terminal HlyA\textsubscript{594-1023} region.

While the four positive reacting MAbs are also reactive in the prelytic state, four additional MAbs react with HlyA\textsuperscript{p} at 0 to 2°C. MAb C10 reacts to an epitope in the N terminus which is accessible at 0 to 2°C, in contrast to its inaccessibility at 23°C. A striking MAb reactivity difference between the lytic and prelytic bound states is the positive reactivity of the B7 epitope at 0 to 2°C, because a directly adjacent epitope with very strong immunoblot and ELISA titers (MAb H10) remains inaccessible at this temperature. Because of its proximity to the B7 epitope, we believe that reactivity to the H10 epitope is occluded by local folding in a manner that results in inaccessibility at both 0 to 2 and 23°C, rather than insertion within the erythrocyte membrane. MAbs B10 and C7, which react against a conformational epitope, are nonreactive at 23°C. MAb C7 does show slight reactivity at this temperature but in an inconsistent manner, suggesting the epitope is within or near a dynamic structure. What is striking is that at 0 to 2°C, both MAbs B10 and MAb C7 consistently react to HlyA\textsuperscript{p}. It is difficult to assess the particular conformational changes associated with the reactivities of MAbs B10 and C7, but we can conclude that these epitopes are stable in the prelytic state of bound toxin. In the lytic state, however, the epitopes are de-
stroied, membrane embedded, or surface exposed but in an inaccessible conformation. We conclude that distinct structural differences exist in the N terminus of the toxin, as well as in the HlyA518–530 region between the prelytic and lytic states of bound toxin. Regions in proximity of epitopes for MAbS E2, H10, A10, and D12 are inaccessible at both experimental temperatures.

We hypothesized that changes in the acylation status of the hemolysin would be reflected by differences in MAb reactivity between the wild-type toxin and different HlyA mutants directly affected in acylation. First, we confirmed the recent observation that nonacylated pro-HlyA does stably associate with erythrocytes (1). The same can be said of the HlyA mutants which are substituted with cysteines at either of the known sites of acylation (HlyAK563C and HlyAK689C). The MAb reactivity profiles for proHlyAE and especially HlyAK563C indicate a generally lower percentage of erythrocytes with MAb-reactive species bound than seen with HlyAE, and at first glance this may be attributed to a general reduced binding to erythrocytes. We do not favor this conclusion, however, because if the HlyAK563C mutant is directly fluoresceinated at the cysteine residue, bound to erythrocytes, and analyzed by flow cytometry, it shows binding levels similar to those for the wild-type toxin (18). Therefore, we prefer an argument that an equal number of mutant toxin molecules bind erythrocytes, but many are simply not detected with antibodies for unknown reasons. We also cannot draw any conclusions on the structural basis for the overall lower reactivity seen with proHlyAE and HlyAK563C compared to HlyAE.

More MAbs react with HlyAK689C at 23°C than with HlyAE. MAbS B7 and D10 react with the HlyAK563C at this temperature but not with HlyAE. It is very interesting that the G8 epitope has poor accessibility at 23°C and is not accessible at 0 to 2°C in both HlyAK563C and HlyAK689C structures, whereas it is readily available for HlyAE at both temperatures. This finding indicates that a change in the covalent structure of HlyA at position 563 or 689 causes a local change in HlyAE structure involving the very N-terminal 12 amino acids. Perhaps the most striking conformational change observed in the middle of the toxin, covering the B9 epitope at HlyAS94–614, is the prelytic conformation at 0 to 2°C, HlyA and either of the lysine substitution mutants react with MAb B9. However, MAb B9 reacts poorly and inconsistently with proHlyAE at 0 to 2°C as well as 23°C.

The B7, C7, and C10 epitope regions show inconsistent reactivities at 0 to 2°C for all three mutants. Inconsistent reactivity is defined as a situation where a MAb shows a different range of reactivities in separate experiments, ranging from no to very high reactivity. These three epitopes were shown to have distinct and stable contrasting reactivities with the wild-type toxin in its prelytic and lytic states. We cannot explain the structural basis for the inconsistency seen in MAb reactivities for these epitopes in the mutants. The only MAbs for which consistent reactivity was seen in all experiments were the nonreactive MAbs A10, E2, D12, and H10.

Proteolytic treatment of wild-type HlyAE prior to testing of MAb reactivities indicated that in most cases, epitopes are totally removed from more than 50% of E3HlyAE, accompanied with 10- to 500-fold reduction in the fluorescence intensity on the remaining E3HlyAE. The level of fluorescence intensity indicates the number of available epitopes per cell. None of the normally nonreactive MAbs H10, E2, D12, and A10 became reactive with proteolytic treatment. A fascinating result seen in these experiments is that the HlyAS94–614 region containing the B9 and G3 epitopes appears resistant to proteolysis at 0 to 2°C. The region containing these epitopes is surface exposed at both temperatures and proteolytically sensitive at 23°C; therefore, it is also expected to be sensitive at 0 to 2°C. These results indicate an altered local folding of this domain at 0 to 2°C. Curiously, MAb G3 reacts to the same HlyAS26–639 region (AGSANIYAGKGHDV) as MAB D1 does. MAb D1, however, can no longer react to proteolytically treated HlyAE.

Based on this difference in proteolytic susceptibility, we hypothesize that the G3 epitope is N terminal to the D1 epitope, with the D1 epitope potentially involving the HlyA563–639 trypsin digestion site.

In recent investigations, we used site-specific fluorescein-labeled cysteine substitution mutants of HlyA in conjunction with flow cytometry to discover that residues 344, 369, 563, 578, 689, 696, and 913 cannot be proteolyzed from HlyAE at either 0 to 2 or 23°C (18). These residues are not within the reactive MAb regions studied here, although two sites (563 and 578) fall between the B9 and B7 epitopes. Both of these epitopes are fully accessible at 0 to 2°C, while B7 is inaccessible at 23°C. The regions covering residues 563 and 578 are also resistant to proteolysis at 0 to 2°C. We conclude that the 563 acylation region, similar to the 689 lysine acylation site, does not become accessible at either temperature, despite the altered conformation and accessibility of the directly N-terminal B7 epitope region at the two temperatures.

The only proteolytically susceptible tested fluorescein-labeled residue was amino acid 131 in the prelytic structure of HlyA5. The epitope for MAB C10 lies close to this site and is also proteolytically removed at 0 to 2°C. In the lytic state, the C10 epitope is nonreactive and the 131 site is not proteolyzed from HlyAE5. These complementary findings provide evidence for distinct changes in the conformation and accessibility of the N terminus of HlyA in its prelytic and lytic membrane-bound conformations and further validate flow cytometry analyses of HlyAE5 topology.

**Model of HlyAE topology.** Based on the results presented here, models of the topology of HlyA in erythrocyte membranes in prelytic and lytic states can be derived, but there are several caveats to be discussed. The lack of MAb reactivity to an epitope present in an integral membrane protein could be the result of occlusion of the epitope by protein folding, modification, or interaction with another protein. These factors are as likely to cause loss of reactivity to an epitope as its insertion into the erythrocyte membrane. These topological constraints could also result in loss of proteolytic sensitivity at any particular site of a membrane-associated protein. For simplicity, our models will describe lack of MAb reactivity or protease sensitivity of a region in terms of protection by membrane insertion, although it is clear that more complicated molecular explanations are likely. Regardless of actual HlyA topology relative to the target membrane, the differences observed in MAb reactivity observed in these investigations for the different states of associated toxin do indicate distinct conformational states. Two main regions of HlyA change in surface accessibility between the prelytic and lytic states. One is the HlyA12–131 region and the other is the HlyA518–530 region.

For the HlyAE lytic state, the results presented here support the hypothesis that the large 350-amino-acid region C terminal to HlyAS131 is membrane inserted. Acylation site HlyAS563 and the region surrounding it are also inserted. Between HlyAS563 and another inserted area surrounding acylation site HlyA689, the HlyA564–640 region is exposed on the external side of the erythrocyte membrane. This region is predicted to have the highest probability of surface exposure by different computer algorithms (21). The lack of A10 reactivity for HlyAE suggests that the calcium binding repeat region is embedded in the membrane. This result is consistent with the
observation made with the small nodO RTX exoprotein (6). The NodO protein essentially consists of only the repeat-like sequence and can insert and form pores in artificial lipid bilayers (6).

The model for the prelytic HlyAE complex predicts two surface-exposed areas which become membrane inserted in a temperature-dependent fashion to achieve the mature lytic conformation of HlyAE. The N-terminal HlyA12–131 region and central HlyA18–530 region are external to the membrane in the prelytic HlyAE state. In the fully competent lytic HlyAE complex, these two regions become embedded within the membrane-toxin structure. The observation that the acylation status of HlyA has conformation consequences in the N-terminal region of HlyA provides striking evidence that acylation has global rather than a simple local impact on the structure and function of hemolysin.

Application of flow cytometry methods in the study of RTX toxins against nonerythrocyte cell types is an important next step in the RTX field. Preliminary studies of the association of HlyA with and its cytotoxicity against Raji and BL-3 cell lines indicate MAb reactivity profiles different from what is reported here for erythrocytes. Additionally, our studies on the association of other RTX toxins with different target cells show a lack of correlation between toxin binding and target cell susceptibility. We believe that the flow cytometry techniques applied in this study represent a powerful new tool for the study of conformations associated with target-bound hemolysin and will help dissect the steps associated with the lytic process induced by this toxin in membranes.

ACKNOWLEDGMENTS

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