A Receptor-binding Region in Escherichia coli α-Haemolysin*

Received for publication, August 21, 2002, and in revised form, February 10, 2003
Published, JBC Papers in Press, February 11, 2003, DOI 10.1074/jbc.M208552200

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Escherichia coli α-haemolysin (HlyA) is a 107-kDa protein toxin with a wide range of mammalian target cells. Previous work has shown that glycophorin is a specific receptor for HlyA in red blood cells (Cortajarena, A. L., Goñi, F. M., and Ostolaza, H. (2001) J. Biol. Chem. 276, 12513–12519). The present study was aimed at identifying the glycophorin-binding region in the toxin. Data in the literature pointed to a short amino acid sequence near the C terminus as a putative receptor-binding domain. Previous sequence analyses of several homologous toxins that belong, like HlyA, to the so-called RTX toxin family revealed a conserved region that corresponded to residues 914–936 of HlyA. We therefore prepared a deletion mutant lacking these residues (HlyA914–936) and found that its hemolytic activity was decreased by 10,000-fold with respect to the wild type. This deletion mutant was virtually unable to bind human and horse red blood cells or to bind pure glycophorin in an affinity column. The peptide Trp914–Arg936 had no lytic activity of its own, but it could bind glycophorin reconstituted in lipid vesicles. Moreover, the peptide Trp914–Arg936 protected red blood cells from hemolysis induced by wild type HlyA. It was concluded that amino acid residues 914–936 constitute a major receptor-binding region in α-haemolysin.

α-Haemolysin (HlyA) is a 107-kDa protein toxin secreted by pathogenic strains of Escherichia coli. It is a member of the so-called “RTX family,” a group of proteins characterized by the presence of a Gly- and Asp-rich nonapeptide sequence repeated in tandem near the protein C terminus (for reviews, see Refs. 1–5). These repeats constitute a Ca2+-binding domain whose structure has been solved at high resolution for a non-toxin member of the RTX family, the alkaline protease from Pseudomonas aeruginosa (6). HlyA first binds a receptor on the cell surface, a β2-integrin receptor in leukocytes (7) or glycophorin in red blood cells (8), and then becomes inserted in the cell membrane. Recent data indicate that insertion may take place in the absence of Ca2+ (9, 10), but Ca2+ binding to the nonapeptide repeat domain is essential for membrane lysis (9, 11, 12). Note that the Ca2+-binding domain is located near the protein C terminus, whereas the membrane insertion domain is located near the N terminus (9, 10, 13).

The present study is devoted to exploring the early stages of HlyA interaction with the target cell, namely its binding to the surface receptor. In particular, our investigation is aimed at the region(s) of the protein that bind(s) the receptor glycophorin on mammalian erythrocytes (8). A number of previous studies suggest that a region between the repeat domain and the C terminus may be involved in binding this specific receptor. For example, Bejerano et al. (14) have described two amino acid “blocks,” located after the nonapeptide repeats, in the C terminus of the adenylate cyclase toxin (another member of the RTX family). Block A (15 amino acids) is essential for the toxic activity since it is required for the toxin binding and insertion into the membrane. Deletion of block B, however, does not affect the toxin activity. HlyA possesses homologous A and B blocks (Fig. 1), and it has been shown (14) that deletion of a region that includes the last two residues of block A, the connection between the blocks, and the first nine amino acids of block B abolishes the hemolytic activity. Previously, Chervaux and Holland (15) had shown that five point mutations in that region (residues 918, 920, 921, 928, and 932) inhibited hemolysis without affecting protein export.

Leukotoxin from Pasteurella hemolytica, also a RTX toxin, can be neutralized by incubation with a specific antibody whose epitope is located in the C-terminal region of leukotoxin between residues 841–872 (16). This epitope appears to be related to the binding of the toxin to the β2-integrin receptor in the target cell (17). The sequence of the epitope overlaps partially blocks A and B: 841-WFREADAKEVNYKATKDEK-IEEIQQNGER972. Another RTX toxin, leukotoxin from Actinobacillus actinomycetemcomitans, contains an epitope, recognized by neutralizing antibodies, that begins 78 residues after the last repeat and overlaps partially with the sequence given above (18). Finally, in the adenylate cyclase toxin, deletion of the last 75 residues abolishes both the lytic and cell binding activities (19). The deleted region contains a sequence homologous to the leukotoxin epitope mentioned above.

In view of the above findings, we performed a sequence analysis on members of the RTX family, searching for peptides homologous to the 841–872 epitope of leukotoxin, with the result that this sequence was found to be highly conserved among members of the family, including HlyA (residues 914–936). A mutant HlyA lacking this peptide (HlyAΔ914–936) was prepared and found to be incapable of binding erythrocytes or purified glycophorin. However, the 914–936 peptide (WR peptide) did bind pure glycophorin and protected red blood cells from HlyA hemolysis. Thus, residues 914–936 of HlyA appear to be essential for the toxin to bind its erythrocyte receptor, glycophorin.

EXPERIMENTAL PROCEDURES

Materials—Horse red blood cells were supplied by Biomedics (Alcobendas, Spain). Human erythrocytes were obtained from a local blood...
bank. Glycophorin from horse or human erythrocyte ghosts was puri-
ified as described previously (8). Egg phosphatidylcholine was Grade I
from Lipid Products (South Nutfield, England). Rhodamine-phosphati-
dylethanolamine was purchased from Avanti Polar lipids (Alabaster,
Alabama). Hi-Trap desalting, Superdex HR-200, and N-hydroxysuccinimide-
activated Hi-Trap columns were supplied by Amersham Biosciences.
Oligonucleotides were synthesized by Amersham Biosciences. 

**Toxin Binding to Phospholipid Vesicles**

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**Wild type HlyA and HlyAΔ914–936 were expressed in an E. coli D1210 strain containing plasmid pSU124 and purified as described by Ostolaza et al. (20) for the wild type. The proteins were stored at −20 °C in 150 mM NaCl, 6 mM urea, 20 mM Tris-HCl, pH 7.0 buffer.**

**Hemolysis Assays**—A standard red blood cell suspension was used, obtained by diluting the erythrocytes with saline so that 37.5 μl of the mixture in 5 ml of distilled water gave an absorbance of 0.6 at 412 nm. Erythrocyte suspensions of the standard suspension of washed human or horse erythrocytes were added to serial 2-fold dilutions of hemolysin in hemolysis buffer (150 mM NaCl, 10 mM CaCl2, 20 mM Tris-HCl, pH 7.0) in a microtiter plate. The mixtures were incubated at room temperature for a few hours so that erythrocyte sedimentation occurred. The absorbance of the supernatants, appropriately diluted with distilled water, was measured at 412 nm. The blank (zero hemolysis) consisted of a mixture of equal volumes of buffer and erythrocytes.

**Toxin Binding to Erythrocytes**—Erythrocytes were washed and re-
suspended in hemolysis buffer at 2 × 107 cells/ml. The appropriate amounts of WT (wild type) or mutant HlyA were added, and the mixture was incubated at 37 °C for 30 min. The cells were then centrifuged at 14,000 × g for 10 min at room temperature. The pellet cells were lysed at 4 °C with 5 mM phosphate buffer, pH 8.0, and washed in the same buffer by centrifugation (14,000 × g, 10 min, 4 °C). The red blood cell membranes were resuspended in the same volume of 4% (w/v) SDS, 4% (w/v) glycerol, 0.02% (w/v) bromophenol blue, 100 mM 4-dimethylam-
inothiol, 50 mM Tris-HCl, pH 6.8 and boiled for 5 min. These samples were subjected to SDS-PAGE and then transferred to nitrocellulose by the method of Towbin et al. (21). Blots were blocked with 10% skim milk in TBST buffer (150 mM NaCl, 0.05% Tween 20 (w/v), 10 mM Tris-HCl, pH 7.5) for 2 h at room temperature. They were then incubated with a solution containing a polyclonal rabbit anti-hemolysin antibody (1:1,000) in 5% skim milk/TBST overnight at 4 °C, washed with TBST buffer, incubated with peroxidase-conjugated anti-rabbit IgG antibody (Sigma) (1:2,000) in TBST buffer with 5% skim milk for 1 h at room temperature. Immunoblots were developed by a chemilumines-
cent method (ECL, Amersham BioSciences).

**Toxin Binding to Phospholipid Vesicles**—Toxin binding to large unilamellar vesicles composed of egg phosphatidylcholine was assayed by the flotation method of Pereira et al. (22). The vesicles were prepared (23) containing 0.6 mole percentage of rhodamine-phosphatidylethanol-
amine and diluted to 250 μM in a D2O buffer (150 mM NaCl, 10 mM CaCl2, 20 mM Tris-HCl, pH 7.0). Liposomes were incubated with protein for 1 h at 25 °C at 1:2,500 and 1:5,000 protein:lipid molar ratios. Then liposome-bound and non-bound proteins were separated by ultracen-
trifugation in a TLA 120.2 Beckman rotor (627,000 × g, 2 h, 20 °C). Liposomes containing bound protein floated on top of the buffer. The upper fraction was removed, liposomes were solubilized in detergent, and lipid and protein were quantitated by fluorescence.

**Toxin Binding to Glycophorin—**An N-hydroxysuccinimide-activated Hi-Trap column (1 ml) from Amersham Biosciences was used to bind purified glycophorin. The column was first washed (3 × 2 ml) with cold 1 mM HCl to remove isopropyl alcohol. Then the purified glycophorin solution (0.5 mg/ml) prepared in the coupling buffer (0.5 mM NaCl, 0.2 mM NaHCO3, pH 9.3) was added and left overnight at 4 °C. The deactiva-
tion of any excess active groups that had not coupled to the lipid protein and the washing out of the nonspecifically bound lipids were performed following exactly the procedure described by the supplier.

**Protection against HlyA-induced Hemolysis by WR Peptide**

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**Analysis of RTX toxin sequences dis-

**Sequence Alignments—**Analysis of RTX toxin sequences dis-
tal to the repeat domain revealed a conserved region analogous to the 841–872 epitope of leukotoxin (Fig. 2). The alignment showed that some high homology sequences coexisted with more variable ones. This may be related to the fact that some RTX toxins (leukotoxins) attack a narrow range of target cells, whereas others (hemolysins) are less specific (3). The homolo-
gous region of HlyA, including residues 914–936, was the object of our experimental studies. It was designated as the WR peptide because of its initial and final amino acid residues.

The HlyA914–936 Deletion Mutant—HlyA914–936 was expressed in E. coli in the same amounts as the WT (~2 mg/liter culture filtrate) and could be purified following the same protocol (20). It was recognized by the same polyclonal antibodies raised against WT HlyA (8). However, its lytic activity on horse or human red blood cells was of ~10^4 times less than the WT (Fig. 3). This is in agreement with the idea that amino acids 914–936 are involved in HlyA binding to its erythrocyte receptor. If this were the case, the hemolysis observed at very high concentrations of HlyA914–936 might be due to secondary receptor-binding domains in the toxin, in agreement with the suggestion by Bauer and Welch (25) that several regions in the protein may be involved in cell binding. Other proposals have been made (26, 27) involving the acylated repeat region of HlyA, including residues 914–936. Deletion of amino acids 914–936, was the object of our experimental studies. It was designated as the WR peptide because of its initial and final amino acid residues.

The WR Peptide

The WR Peptide—The WR peptide, representing residues
914–936 of HlyA, was synthesized and used in lysis and binding assays. These experiments were designed as a complement to those performed with the proteins lacking precisely this peptide. The WR peptide had no hemolytic activity of its own, even when assayed at very high concentrations (Fig. 6).

To confirm that the region deleted in HlyAΔ914–936 participates in receptor binding, binding of the deleted WR peptide to glycophorin reconstituted in liposomes was assayed. For this purpose, phospholipid (egg phosphatidylcholine) vesicles were prepared containing glycophorin, as described in Ref. 24. A fraction of these vesicles eluting as a symmetric peak from a Sepharose 2B-300 column was analyzed for protein and lipid P and found to contain phosphatidylcholine and glycophorin at a ~1,000:1 mole ratio. Protein-free large unilamellar vesicles composed of pure egg phosphatidylcholine were used as a control. Binding of WR peptide to reconstituted glycophorin was assessed through changes in the intrinsic Trp fluorescence of the peptide as a function of lipid concentration. Trp fluorescence increases when the peptide in solution binds the less polar membrane environment. The results in Fig. 7 demonstrate that WR peptide binds large unilamellar vesicles containing glycophorin, but does not bind to unilamellar vesicles not containing glycophorin.

Protection by WR Peptide of HlyA-induced Hemolysis—In the absence of a direct proof of specific binding of WR peptide to glycophorin in erythrocytes, we have observed that pretreatment of red blood cells with the peptide decreases the extent of
hemolysis induced by HlyA. The dose-dependent inhibition of hemolysis by the WR peptide is shown in Fig. 8. These data suggest that the peptide is binding the erythrocytes and subsequently preventing the specific binding of HlyA. Thus, this peptide would appear to be binding the specific receptor glycoporphin.

Conclusions—From the above experiments, we conclude that the HlyA region corresponding to amino acids 914–936 is a major determinant in the specific binding of HlyA to the red blood cell surface through glycoporphin. The fact that a peptide containing amino acids 914–936 provides protection against an HlyA challenge may suggest a therapeutic application of this peptide in the extraintestinal E. coli infections in which HlyA has a pathogenic role. Moreover, homologous sequences of the WR peptide exist in other RTX toxins; thus, the results in this study can probably be extended to those toxins as well.

Acknowledgments—The authors are indebted to the Instituto de Immunologia de Colombia for the generous gift of the WR peptide and to Professor R. N. McElhaney (University of Alberta) for reading and improving the manuscript.

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