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A peptide derived from the putative transmembrane domain in the tail region of *E. coli* toxin hemolysin E assembles in phospholipid membrane and exhibits lytic activity to human red blood cells: Plausible implications in the toxic activity of the protein

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ABSTRACT

Hemolysin E (HlyE), a pore-forming protein-toxin and a potential virulence factor of *Escherichia coli*, exhibits cytotoxic activity to mammalian cells. However, very little is known about how the different individual segments contribute in the toxic activity of the protein. Toward this end, the role of a 33-residue segment comprising the amino acid region 88 to 120, which contains the putative transmembrane domain in the tail region of HlyE has been addressed in the toxic activity of the protein-toxin by characterizing the related wild type and mutant peptides and the whole protein. Along with the 33-residue wild type peptide, H-88, two mutants of the same size were synthesized; in one mutant a conserved valine at 89th position was replaced by aspartic acid and in the other both glycine and valine at the 88th and 89th positions were substituted by aspartic acid residues. These mutations were also incorporated in the whole toxin HlyE. Results showed that only H-88 but not its mutants permeabilized both lipid vesicles and human red blood cells (hRBCs). Interestingly, while H-88 exhibited a moderate lytic activity to human red blood cells, the mutants were not active. Drastic reduction in the depolarization of hRBCs and hemolytic activity of the whole toxin HlyE was also observed as a result of the same double and single amino acid substitution in it. The results indicate an important role of the amino acid segment 88–120, containing the putative transmembrane domain of the tail region of the toxin in the toxic activity of hemolysin E.

1. Introduction

Gram-negative bacteria often produce a variety of cytolytic toxins, which enable the microorganisms to successfully infect the host including humans and other animals [1–3]. However, the exact mode of actions and involvement of the majority of these cytolytic toxins in the pathogenesis of infections are not clearly understood. A number of cytolysins isolated from gram-negative bacteria form pores in the cytoplasmic membranes of eukaryotic cells [4,5]. Disease causing strains of *Escherichia coli* (*E. coli*) produce several pore-forming toxins. Of these, α-hemolysin (HlyA) is found in *E. coli* strains, which cause extra-intestinal infections, while enterohemorrhagic *E. coli* (EHEC) hemolysin is produced by EHEC strains of serogroup O157 [2,6]. The nonpathogenic strain, *E. coli* K-12, does not contain the gene cluster required for the production and secretion of α-hemolysin or the related EHEC hemolysin and is non-hemolytic under normal condition. However, a variant of *E. coli* K-12 can also express a hemolytic or cytolytic phenotype under certain conditions. This induction of cytolytic activity in the laboratory strain, *E. coli* K-12 has been shown to be due to the synthesis of a toxin, named as hemolysin E (HlyE) or silent hemolysin A (SheA) or cytolysin A (ClyA) by different groups [7,8]. *E. coli,* expressing hemolysin E can cause lysis of red blood cells from different species, including humans, horse, sheep, goat and hen [8–11]. The expression of hlyE gene in *E. coli* K-12 is silenced by the nucleoid protein histone-like nucleoid-structuring protein (H-NS) [12] while it is activated in the presence of overproduction of SlyA, MprA, HlyX or fumarate and nitrate reduction regulator (FNR) [8,9,12,13]. The expression of hemolysin E has also been found in clinical isolates of *E. coli* [14]. Furthermore, purified HlyE and HlyE-expressing *E. coli* exhibit cytotoxic and apoptogenic activities towards human and murine macrophages, human

Abbreviations: HlyE, hemolysin E; TFE, Trifluoroethanol; NBD-fluoride, 4-fluoro-7-nitrobenz-2-oxa-1, 3-diazole; Rho, Tetramethylrhodamine; PC, Egg phosphatidylcholine; PG, Egg phosphatidylglycerol; Chol, Cholesterol; a.a., amino acid; hRBC, human red blood cell

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peripheral monocytes and HeLa cells [15]. Recent studies have shown that hemolysin E after secretion into the periplasm of *E. coli,* is packaged in its outer membrane vesicles in the active form and from there transported into the target mammalian cells [16].

Crystal structure of hemolysin E has been solved at 2.0 Å resolution, which indicated that it belongs to a new family of toxin structures [17]. A structure–function study showed that the deletion of 37 amino acids from the αG region, at the C-terminal of the toxin rendered it non-hemolytic [18]. An amphipathic, conserved leucine zipper motif from hemolysin E has been identified and characterized [19–21], which suggested a possible structural and functional role of this motif in the toxin. However, it is still not known how hemolysin E exhibits cytotoxic activity to eukaryotic cells for example lyses human red blood cells. The literature shows that for a number of proteins belonging to several families like transcription factors, fusion proteins of enveloped virus and pore-forming toxins it has been possible to identify small segments that show certain activity of the corresponding whole protein. However, for hemolysin E no such segment with hemolytic activity of the toxin was known.

With a goal to understand the contribution of different conserved hemolysin E segments to the membrane-interaction and functional activity of the whole protein, we have synthesized and characterized a 33-residue peptide (H-88, amino acid 88–120), which contains a putative transmembrane domain [13,17], located in the tail region of the protein along with two mutants of the same size. In one of them (Mu1-H-88), a conserved valine at 89th position was substituted by an aspartic acid while in the other glycine and valine positioned at the 88th and 89th positions were replaced by two aspartic acid residues. The lytic activity of *E. coli* containing the hemolysin E plasmid with the latter mutations was significantly reduced [9]. Therefore, it was of interest to look into the effect of the same mutation on the activity of the H-88 segment. In the first mutant the purpose was to look into the effect of the substitution of the single amino acid positioned in the putative transmembrane segment. Furthermore, in order to look into the effect of these single and double amino acid substitutions on the cytotoxic activity of the whole toxin, site directed mutagenesis was performed in the hlyE plasmid to prepare both the mutant proteins with the above amino acid substitutions. The wild type and mutant proteins were purified and then characterized along with the synthetic peptides. Results showed that H-88 permeabilized both phospholipid vesicles and human red blood cells (hRBCs), and more interestingly lysed them like the whole protein though with lesser efficiency. Although the amino acid substitutions were done at the N-terminal of the selected segment, the mutant peptides did not exhibit any appreciable pore-forming activity in lipid vesicles or hRBCs and showed no lytic activity toward the hRBCs. Hemolysin E mutants with the same mutations as in the peptide showed drastically reduced hemolytic activity and permeabilized the human red blood cells negligibly as compared to the wild type protein. The results have been discussed in terms of the plausible role of the H-88 segment containing the conserved putative transmembrane region in toxic activity of the protein toxin HlyE.

2. Materials and methods

2.1. Materials

Rink amide MBHA resin (loading capacity, 0.63 mmol/g) and all the N-α Fmoc and necessary side-chain protected amino acids were purchased from Novabiochem, Switzerland. Coupling reagents for peptide synthesis like 1-hydroxybenzotriazole (HOBT), di-isopropyl-carbodiimide (DIC), 1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and N, N′-diisopropylethylamine (DIPEA) were purchased from Sigma, USA. Dichloromethane, N, N′ dimethylformamide (DMF) and piperidine were of standard grades and procured from reputed local companies like Merck Limited, Mumbai, Thomas Baker, Mumbai and Spectrochem Pvt. Ltd., Mumbai respectively. Acetonitrile (HPLC grade) was procured from Merck, India while trifluoroacetic acid (TFA), trifluoroethanol (TFE) and sodium dodecyl sulfate (SDS) were purchased from Sigma. Egg phosphatidylcholine (PC) and egg phosphatidylglycerol (PG) were obtained from Northern Lipids Inc., Canada while cholesterol (Chol) was purchased from Sigma. NBD-fluoride (4-fluoro-7-nitrobenz-2-oxa-1, 3-diazole) and tetramethylrhodamine succinimidyl ester were procured from Molecular probes (Eugene, OR) whereas calcein was purchased from Sigma and used without any further purification. Rests of the reagents were of analytical grade and procured locally; buffers were prepared in milli Q water.

2.2. Peptide synthesis, fluorescent labeling and purification

All the peptides were synthesized manually on solid phase. Stepwise solid phase syntheses were carried out on rink amide MBHA resin (0.15 mmol) utilizing the standard Fmoc chemistry and employing DIC/HOBT or TBTU/HOBT/DIPEA coupling procedure [22] as already reported [19,23]. De-protection of α-amino group and the coupling of amino acids were checked by the Kaiser test [24] for primary amines. After the synthesis, each peptide was cleaved from the resin with simultaneous de-protection of side chains by treatment with a mixture of TFA/phenol/thioanisole/1,2 ethanedithiol/water (82.5:5:5:2.5:5 v/v) for 6–7 h. Labeling at the N-terminus of a peptide with NBD or rhodamine was achieved by standard procedures as reported [19,25,26]. After sufficient labeling, the resins were washed with DMF and DCM in order to remove the un-reacted probe. The peptides were cleaved from the resin as above and precipitated with dry ether. All the labeled and unlabeled peptides were purified by reversed-phase HPLC on an analytical Vydac C4 column using a linear gradient of 0–80% acetonitrile in 45 min with a flow rate of 0.8 ml/min. Both acetonitrile and water contained 0.05% TFA. The purified peptides were ~95% homogeneous as shown by HPLC. Experimental mass of the peptides as detected by ES–MS analysis corresponded very close to their theoretical values.

2.3. Generation of double and point mutation in hlyE

Site directed mutagenesis was done in order to substitute Glycine at 88th and Valine at 89th position by aspartic acid residues in HlyE. This was accomplished by introducing mutations in the internal forward FPIHlymE (5' AT GAA TGG TGT GAT GAT GCG ACG CA 3') and reverse primer RPIHlymE (5' TG CGT CGC ATC ATC ACA CCA TTC AT 3') as underlined. The PCR was done using pGS-1111 (construct of pGEX-KG and hlyE) as the template, which was isolated from the engineered *E. coli* JM109 cells (a gift from Prof. Jefrey Green, University of Sheffield, U.K.) carrying the same plasmid using two sets of primer combinations, forward outer FPOHlymE (5'CCA TGG CTG AAA TCG TTG CAG A 3') and internal reverse RPIHlymE and internal forward FPIHlymE and outer reverse RPOHlymE (5'GTC GAC TCA GAC TTC AGG TAC CTC AAA G 3'). The amplified products were gel eluted, quantified and mixed together in equal proportion and further used as template to amplify the full-length hlyE gene using primer set FPOHlymE and RPOHlymE. The amplified hlyE was gel eluted and cloned in pDrive (Quiagen PCR cloning kit), which was subcloned in the expression vector pGEX-KG at NcoI/SalI site as pDKJ1. The positive clone was sequenced on ABI sequencer (Supplementary Fig. 1) and aligned with the original hlyE sequence using Gene Tool software.

The same procedure was followed to introduce the point mutation to replace valine at 89th position by a single aspartic acid residue as mentioned above except the internal primers set used for introducing mutation was replaced by FPIHlymVD (5' AT GAA TGG TGT GGT GAT GCG ACG CA 3') and RPIHlymVD(5' TG CGT CGC ATC ACC ACA CCA TTC AT 3'). The mutated gene was subcloned in the expression vector