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N-terminus of mature heat-labile enterotoxin chain B is critical for its extracellular secretion in *Vibrio cholerae*

Reema Mukhija, Lalit C. Garg*

Gene Regulation Laboratory, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 110067, India

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Abstract The effects of addition of a few amino acids to the amino- and carboxy-terminal regions of the mature portion of the heat-labile enterotoxin chain B (LTB) of *Escherichia coli* on protein export, secretion and assembly were investigated. In *E. coli*, LTB (secretory protein) with or without the extension at the N- or C-terminus accumulated in the periplasmic fraction. For *Vibrio cholerae*, LTB with the extension at the C-terminus was exported to the periplasm followed by secretion to the extracellular milieu. However, LTB with the N-terminus extension was exported to the periplasm only. Our findings suggest that in the case of *V. cholerae*, the N-terminus of the mature LTB plays an important role in its secretion to the extracellular milieu.

Key words: Heat-labile enterotoxin chain B; Extracellular milieu; Periplasmic expression; Secretory expression; N-terminus

1. Introduction

Synthesis of all prokaryotic secretory proteins usually takes place in the cytoplasm and the targeting information is provided by an N-terminal signal peptide. In *Escherichia coli*, many recombinant proteins have been secreted into the periplasm by fusion to a prokaryotic signal sequences [1,2]. However, such targeting sequences are not always enough to mediate the secretion of any cargo polypeptide. This fact suggests that some domains of the mature protein either contribute to or restrict the secretory process. In the case of *E. coli*, true secretion is rare; most of the proteins are exported only to the periplasmic space. Unlike *E. coli*, *Vibrio cholerae* cells secrete several proteins, such as cholera toxin and proteases, into the extracellular milieu [3].

High specificity in the recognition of proteins for the extracellular secretion is also evident from the fact that only certain proteins are translocated from the periplasm through the outer membrane. Periplasmic proteins, such as β-lactamase or alkaline phosphatase, remain periplasmic if expressed in *V. cholerae*. At present it is not clear how the targeting specificity for extracellular secretion is realised [4]. This process probably involves recognition of specific transport signals by the transport machinery. The signals are likely present in secreted proteins and absent in non-secreted proteins. We suggest that *V. cholerae* can be used as a model system for understanding the mechanism of protein secretion of true extracellular proteins by Gram-negative bacteria.

The heat-labile enterotoxin B chain (LTB) was used as a reporter gene to study the mechanisms of protein exocytosis, assembly of multimeric protein complexes, and secretion of proteins into the extracellular milieu of *V. cholerae* [5–8]. Heat-labile enterotoxin (LT) of *E. coli* and cholera toxin (CT) of *V. cholerae* both represent a doughnut-shaped pentagonal ring, formed by five 11.6-kDa B subunits to which one A subunit (27 kDa) is attached via an extended C-terminal fragment to a region within the central pore of the B subunit pentamer [9–12]. Both the A and B subunits of CT and LT are synthesised as precursor proteins. After translocation across the cytoplasmic membrane and removal of leader peptides, assembly of the subunits (or of the B subunits alone, if the A subunit is absent) occurs in the periplasm [13–15]. *V. cholerae* and certain other Gram-negative species have a specific secretory mechanism, providing protein translocation across the cell envelope. Although several *esp* genes essential for extracellular secretion from *V. cholerae* have been identified, the mechanism of protein translocation across the outer membrane is still unclear [16,17].

Deletion, addition or mutation of random nucleotides at the 3′ end of the LTB gene have been thoroughly investigated and have been shown to affect the export, secretion and assembly of LTB [18–20]. However, little is known about the effects of alterations in the N-terminus of LTB on its export into the periplasm and extracellular secretion. In this study, we report that the addition of amino acids to the N-terminus of mature LTB causes a defect only in its secretion from the periplasm to the extracellular milieu of *V. cholerae*. However, its export to the periplasmic space, assembly into pentamers and binding to G_{M1} ganglioside receptors remain unaffected.

2. Materials and methods

2.1. Bacterial strains and growth conditions

V. cholerae JBK70 (A^-B^-) cells and plasmid pMMB68 were kindly provided by Dr M. Levine and Dr J. Holmgren, respectively. DH5α cells harbouring the plasmid were grown in Luria-Bertani (LB) medium with ampicillin (100 μg/ml) and *V. cholerae* (JBK70) cells containing the plasmid were grown with ampicillin (100 μg/ml) and polymyxin B (50 U/ml) at 37°C on a gyratory shaker. Isopropyl-β-D-thiogalactopyranoside (IPTG) at a concentration of 1 mM was added to the culture at an optical density of 0.5 at 600 nm, and cells were harvested after 8 h.

2.2. Construction of plasmids

The master clone (p18SSHis) harbouring the full-length LTB signal sequence (LTBss) [18] and histidine tag is depicted in Fig. 1A. Genes of interest could be cloned either at the N-terminus at the *Nhe*I site or at the C-terminus at the *Sac*I and *Hin*dIII sites. To clone LTB with the N-terminal extension containing the His tag, a *Sac*I-*Hin*dIII frag-

*Corresponding author. Fax: (91) (11) 6162125.
E-mail: lgarg@hotmail.com or lalit@nii.res.in

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ment encompassing the full-length LTB cDNA (derived from the digestion of plasmid pMMB68) [18] was ligated to *Sac*I- and *Hin*dIII-digested p18SSHis. The *Eco*RI-*Hin*dIII fragment of pl8SSHisLTB was cloned into expression vector pGGhGH-E [21] digested with *Eco*RI and *Hin*dIII. The clone pRMSSHisLTB (Fig. 1B) thus obtained has an LTB with N-terminus extension under the control of the *tac* promoter and can be conjugally transferred to *V. cholerae* cells. To clone LTB with the C-terminal tag, oligos were synthesised to PCR amplify LTB with a *Nhe*I site at both the ends. The sequence of the forward primer is 5′-GCAGCTAGCCCTCAGTCTATTACAG-3′ and that of the reverse primer is 5′-GTAGCTAGCGlTTTCCATACTG-3′. The PCR-amplified LTB fragment was digested with *Nhe*I and cloned into *Nhe*I-digested p18SSHis resulting in LTB with a C-terminal tag. The *Eco*RI and *Hin*dIII fragment of the resultant plasmid p18SHLTB was ligated to *Eco*RI-*Hin*dIII-digested vector pGGhGH-E and the final clone was pRMSHLTB (Fig. 1B). Thus pRMSHLTB is similar to pRMSSHisLTB except for the fact that the amino acid extension is at the C-terminus of LTB.

2.3. Recombinant DNA methods

All restriction and modification enzymes were purchased from New England Biolabs, USA and used as per the recommendations. IPTG was obtained from Boehringer Mannheim, Germany. Isolation of plasmid DNA, digestion of DNA with restriction enzymes, transformation, DNA ligation and agarose gel electrophoresis were performed according to standard molecular biology techniques [22]. DNA fragments were purified by using Gene Clean (Bio 101, Canada). Plasmids pMMB68, pRMSSHisLTB and pRMSHLTB were conjugally transferred to JBK70 cells as previously described [23]. PCR amplification of LTB was conducted using Taq DNA polymerase, as instructed by the manufacturer (Promega, USA), in a Perkin-Elmer thermal cycler.

2.4. Preparation of cellular fractions

The periplasmic fractions were prepared as described earlier [24]. The cell supernatant was concentrated three times by speed vac (Savant, USA). Total cell extract was prepared by suspending 5×10^8 cells in 0.1 M Tris-HCl, pH 8.0 and sonicating at 400 W for 1 min cooling. It was spun at 12 000 rpm for 20 min and the supernatant thus obtained served as total cell extract.

2.5. Western blot analysis and G_{M1} ELISA

For electrophoresis, the samples were boiled in the reducing sample buffer prior to loading. Non-reduced samples were not boiled and were mixed with sample buffer lacking SDS and β-mercaptoethanol. The cellular fractions were electrophoresed on 15% SDS-PAGE [25] and resolved proteins were electrotransferred onto a nitrocellulose membrane for 10 h at 30 mA. Goat anti-LTB polyclonal antiserum (provided by the reagent bank of the National Institute of Immunology, India) was used as primary antibody and anti-goat IgG horseradish peroxidase conjugate was used as secondary antibody. The immunoreactive bands were visualised using 4-chloro-1-naphthol.

Protein estimation was carried out by BCA Protein Assay Reagent kit (Pierce, USA). The concentration of the LTB in different cellular fractions was determined by G_{M1} ganglioside-enzyme linked immunosorbent assay (G_{M1}-ELISA) [26].

3. Results and discussion

3.1. Analysis of expression and secretion of tagged LTB in E. coli

A schematic representation of the clones used in this study is given in Fig. 1B. pMMB68 encoded unchanged LTB with its own LTB signal sequence. pRMSSHisLTB and pRMSHLTB encoded modified LTB with extra amino acids at the N- and C-terminus of LTB respectively. LTB was expressed under the control of inducible *tac* promoter which provided tight control and effective induction with IPTG. Periplasmic fractions prepared from the *E. coli* cells harbouring pMMB68, pRMSSHisLTB and pRMSHLTB were analysed, and the results are shown in Fig. 2. The fractions prepared from the induced *E. coli* cells containing pMMB68 served as positive controls. A band corresponding to the expected size of the LTB monomer was detected in the heat-treated periplasmic fractions of all three clones by immuno-

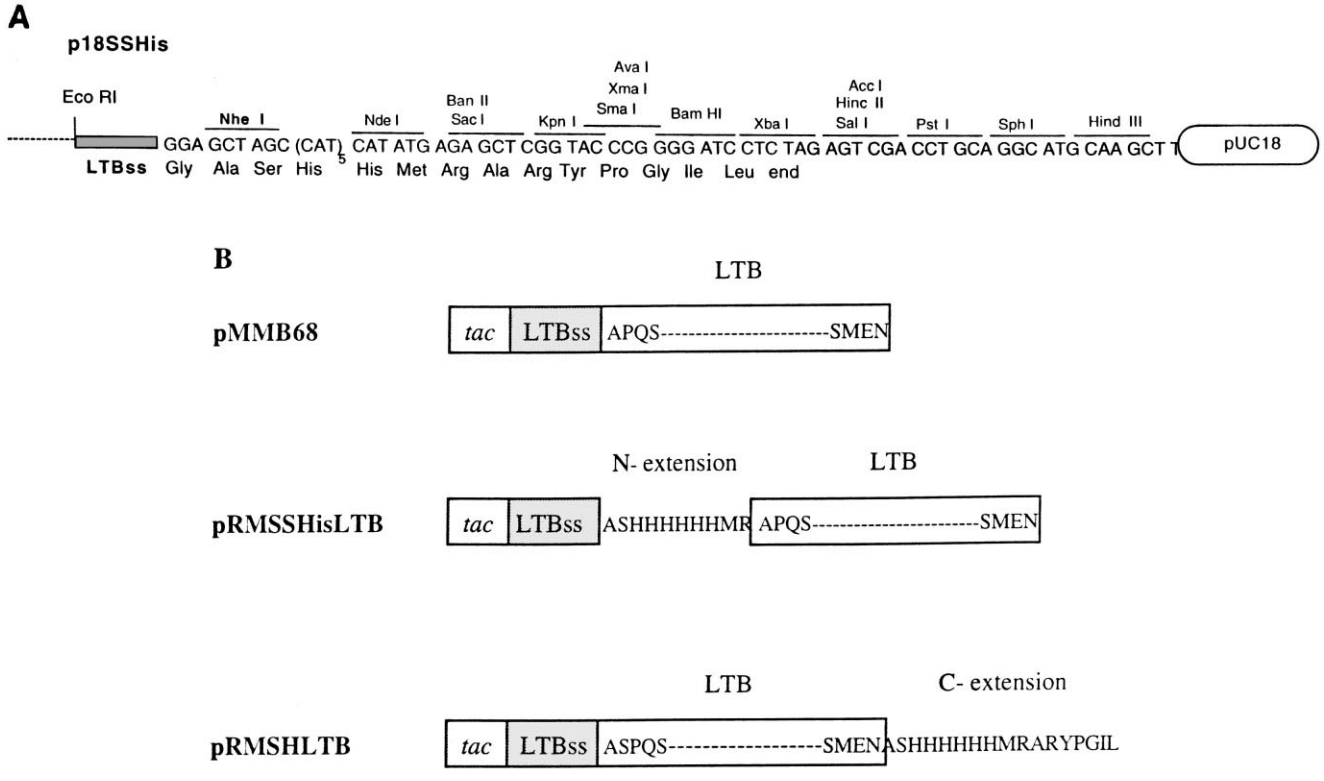

Fig. 1. Schematic representation of the master clone, p18SSHis (A), and clones used in this study (B). His, Histidine tag; *tac, tac* promoter; LTB, heat-labile enterotoxin chain B; ss, signal sequence.

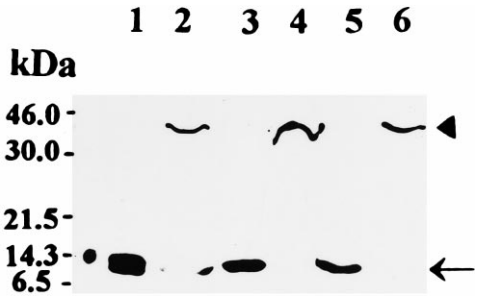

Fig. 2. Analysis of expression of pMMB68, pRMSHLTB and pRMSSHisLTB in *E. coli*. Periplasmic fractions were prepared and analysed using anti-LTB antibodies by Western blotting. Lanes 1, 3 and 5 represent heat-treated periplasmic fractions prepared from cells harbouring plasmid pMMB68, pRMSSHisLTB and pRMSHLTB, respectively, whereas lanes 2, 4 and 6 represent periplasmic fractions without heat treatment. Arrow points to the monomer whereas the arrowhead points to the pentamer of LTB. kDa indicates markers run in parallel with the gel.

blotting using anti-LTB antibodies (Fig. 2, lanes 1, 3 and 5). However, when these fractions were analysed without heat treatment, a band corresponding to LTB pentamer was detected (Fig. 2, lanes 2, 4 and 6). This implies that those amino acid extensions at the N- or C-termini of LTB do not affect its export to the periplasm and do not interfere with its oligomerisation into pentamers.

The secretion of LTB in *E. coli* is known to follow a type II general secretory pathway and it accumulates in the periplasmic space. The transport across the cytoplasmic membrane is accomplished by the *Sec*YEG protein complex. The proteins undergo folding and oligomerisation in the periplasm before translocation occurs through the outer membrane [27]. As expected, translocation of LTB and its modified forms into the extracellular milieu of *E. coli* could not be detected (data not shown). In *E. coli*, it is well known that the final destination of LTB is the periplasmic space. As Fig. 2 shows, the *Sec* machinery does not differentiate the three forms of LTB, and all the forms are secreted to the periplasm in native conformations, which follows from their ability to form pentamers under non-heating conditions. This finding is supported by other studies, in which a C-terminal extension of LTB did not affect its export to periplasm as well as the pentamer formation [5]. An extensive study conducted by Sandkvist et al. [18] has shown that some C-terminal extensions affected neither secretion nor oligomerisation of LTB, whereas some others affected both processes. They also demonstrated that deletion or mutations of amino acids at the C-terminus of LTB have a severe effect on its secretion, oligomerisation and its interaction with the A subunit [19]. In another study, a mutation in the mature part of neutral metalloprotease affected its function with no change in secretion [28].

3.2. Analysis of expression and secretion of His-tagged LTB in *V. cholerae*

pRMSSHisLTB and pRMSHLTB were conjugally transferred into *Vibrio* cells JBK70 A^-B^-. *Vibrio* cells containing these plasmids were induced with 1 mM IPTG for 8 h, and different cellular fractions were analysed for the presence of LTB. *Vibrio* cells containing pMMB68 were used as positive control. When periplasmic fractions containing LTB with or without amino acid extensions at the N- or C-terminus were analysed with or without heat treatment bands corresponding to the LTB monomer or pentamer were detected, respectively (Fig. 3A, lanes 1–3; Fig. 3B, lanes 1–3). In the supernatant of *V. cholerae* cells harbouring pMMB68 and pRMSHLTB, LTB monomer and LTB pentamer were detected under the same conditions (Fig. 3C, lanes 1 and 2; Fig. 3D, lanes 1 and 2). It is evident from the data that the modification of the C-terminus of LTB reduces its secretion into the milieu to some extent. However, LTB was detected neither as a monomer (Fig. 3C, lane 3) nor as a pentamer (Fig. 3D, lane 3) in the supernatant of *V. cholerae* cells containing pRMSSHisLTB. This indicates that amino acid extension at the N-terminus of LTB hinders its translocation from the periplasm of *Vibrio* cells to the extracellular milieu.

Several years ago, Sandkvist et al. [18] showed that the LTB with seven extra amino acids at the C-terminus was secreted from the *V. cholerae* cells as efficiently as the authentic B subunit. Our data imply that the N-terminus of LTB plays an important role in its secretion into the extracellular milieu of *V. cholerae*. Charged residues placed at the N-terminus of inner membrane protein leader peptidase of *E. coli* prevent translocation of the protein across the membrane [29]. The kinetics of streptokinase transport from *E. coli* revealed that the translocation of protein is 3–4 times faster when the first 13 N-terminal amino acid residues of the protein are intact [30]. Yoshihisa and Ito [31] have shown that a pro-OmpA derivative with a His_6 tag in the N-terminal region of the immature protein could not be translocated in the presence

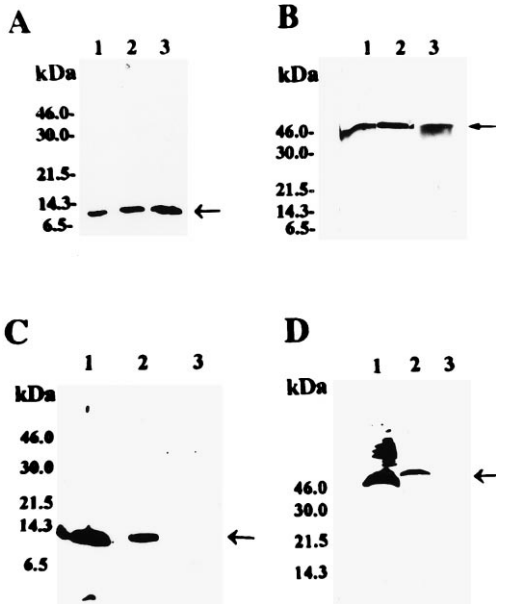

Fig. 3. Western blot analysis of LTB production in *V. cholerae*. Different cellular fractions prepared from *V. cholerae* cells harbouring pMMB68, pRMSHLTB and pRMSSHisLTB were analysed by immunoblotting using anti-LTB antibodies. A: Heat-denatured periplasmic fractions prepared from *V. cholerae* cells harbouring pMMB68 (lane 1), pRMSHLTB (lane 2) and pRMSSHisLTB (lane 3). B: Periplasmic fractions prepared from *V. cholerae* cells were analysed without heat treatment. Lane 1, pMMB68; lane 2, pRMSHLTB; lane 3, pRMSSHisLTB. C: Heat-denatured supernatant fractions prepared from *V. cholerae* cells harbouring pMMB68 (lane 1), pRMSHLTB (lane 2) and pRMSSHisLTB (lane 3). D: Supernatant fractions prepared from *V. cholerae* cells harbouring pMMB68 (lane 1), pRMSHLTB (lane 2) and pRMSSHisLTB (lane 3) analysed without heat treatment. kDa in all the panels indicates markers run in parallel with the gel. Arrow points to LTB.

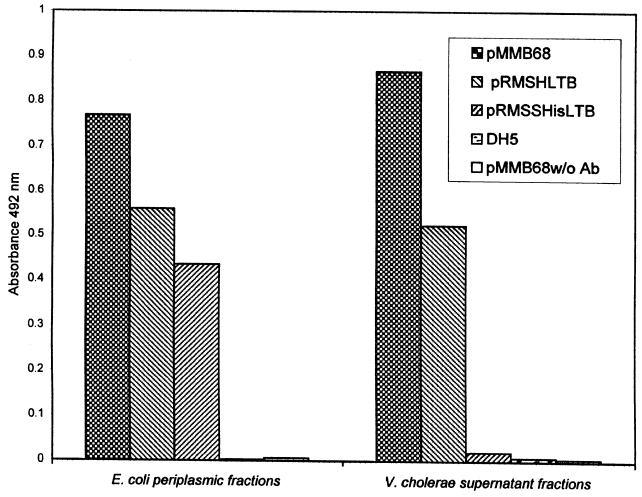

Fig. 4. G_{M1} ganglioside binding activity of LTB in *E. coli* and *V. cholerae* cells harbouring pMMB68, pRMSHLTB and pRMSSHisLTB. In the case of *E. coli*, the periplasmic fractions were analysed whereas in the case of *V. cholerae* culture supernatants were analysed. The ability of LTB to bind to its receptor G_{M1} ganglioside was tested using an in vitro sandwich ELISA. *E. coli* DH5α cells (DH5) and the reactant containing LTB devoid of first antibody (pMMB68 w/o Ab) were used as negative controls.

of Ni^{2+}, while an OmpA derivative with a His_6 tag in the middle or at the C-terminus did not show such Ni^{2+} sensitivity. These studies suggest that the N-terminal domain of the protein is important for maintaining its export-competent status in the translocation process. It is likely that the LTB pentamer contains structural information which is mediated by the N-terminal part of LTB for interacting with the *eps* machinery for its translocation across the outer membrane. Protonation of the N-terminal alanine residue has been shown to affect the assembly of LTB [32].

3.3. Analysis of receptor binding activity

The periplasmic fractions of *E. coli* cells harbouring pMMB68, pRMSSHisLTB and pRMSHLTB were tested for receptor binding activity of LTB using G_{M1} sandwich ELISA. LTB expressed by these three clones was able to bind with the G_{M1} ganglioside receptor (Fig. 4). The periplasmic fraction of *E. coli* cells harbouring pRMSHLTB or pRMSSHisLTB has lower levels of LTB as compared to *E. coli* cells containing pMMB68. Amino acid extensions present at either end of the LTB down-regulated the expression of LTB to a certain extent. However, the effect was more evident in the case when the tag was located at the N-terminus of LTB. Similar results were obtained with periplasmic fractions from *V. cholerae* cells harbouring these plasmids (data not shown).

The supernatants of *Vibrio* cells harbouring pMMB68, pRMSSHisLTB and pRMSHLTB were analysed for G_{M1} ganglioside receptor binding. Those expressing pMMB68 and pRMSHLTB exhibited G_{M1} ganglioside receptor binding activity, whereas that of *V. cholerae* cells expressing pRMSSHisLTB showed no such activity (Fig. 4). These data indicate that the N-terminal extension inhibits the translocation of LTB across the outer membrane completely.

Many of the steps in the export of LTB are presumably common in both *V. cholerae* and *E. coli* including, for example, synthesis of subunit precursors, translocation across the cytoplasmic membrane, maturation, and subunit assembly [33]. The capacity of *V. cholerae* to secrete LTB into the extracellular milieu is, however, an additional step not found in the export of LTB in *E. coli*. It is evident from our studies that N- or C-terminal extension of LTB does not affect its export into the periplasm of *E. coli* and *V. cholerae*, the pathway being similar in both organisms. However, the N-terminal extension inhibited its further transfer into the extracellular milieu of *V. cholerae* suggesting that the N-terminal part of the mature LTB plays an important role in its translocation across the outer membrane of *V. cholerae*.

Considerable efforts have been made to develop improved procedures for the oral delivery of vaccines, one of which is coupling antigens to the non-toxic B subunit of LT, a safe and highly immunogenic protein in humans. Antigens can be coupled to LTB to generate strong mucosal IgA antibody responses to the desired antigen [5,34,35]. The findings of our study suggest that fusions that are made at an inappropriate location may lead to the change in confirmation of LTB, which may not be recognised by the transport machinery of *V. cholerae* and thus inhibiting its extracellular secretion, critical for producing fusion proteins in large amounts for vaccine purpose.

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