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C-terminal glycine-histidine tagging of the outer membrane protein Igaβ of *Neisseria gonorrhoeae*

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Abstract

A glycine-histidine tag (Gly_3His_6) was added to the C-terminus of a fusion protein consisting of the cholera toxin B-subunit (CtxB) and the IgA protease β-domain (Igaβ). The aim was to facilitate single-step purification and to create a suitable tool for kinetic and structural studies on Igaβ-driven protein translocation across the outer membrane of Gram-negative bacteria. We demonstrate that the glycine-histidine tag does not interfere with the assembly of Igaβ in the outer membrane and that the translocator function of the modified Igaβ is maintained. The applicability of the new construct for the dissection of the Igaβ mediated translocation process and general aspects of C-terminal histidine tagging of outer membrane proteins are discussed.

Keywords: *Neisseria gonorrhoeae*; Outer membrane protein; Protein translocation; Igaβ; Histidine tag

1. Introduction

The immunoglobulin A1 protease of *N. gonorrhoeae* MS11 represents the archetypical example of protein secretion in Gram-negative bacteria which includes a self-promoted translocation event across the outer membrane [1]. IgA protease is synthesized as a 169 kDa precursor polyprotein and consists of a typical N-terminal signal peptide, the protease domain (IgaP), and the Igaγ-, the Igaα- and the C-terminal Igaβ domain [2]. The cleavable signal peptide initiates the transport of the precursor across the cytoplasmic membrane probably via the Sec-dependent pathway, while Igaβ seems to be the only factor required for the translocation of the IgaP$\gamma\alpha$-domains across the outer membrane. Following the latter translocation event, auto-proteolytic processing at three different sites of the precursor leads to the extracellular release of the IgA protease, the α-protein and the γ-peptide [2].

The translocation capability of Igaβ is not restricted to its authentic protein passenger, i.e. the IgaPga polyprotein. Rather we could show by attaching the CtxB subunit [3–5] and recombinant antibody fragments (Krämer et al., in preparation) to Igaβ that heterologous proteins can be efficiently translocated across the outer membrane. Igaβ has many features in common with classical outer membrane proteins of Gram-negative bacteria [3,6,7] and appears to assemble in the outer membrane prior to

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promoting the surface-exposure of the respective passenger [4,5]. Igaβ therefore represents a model protein for outer membrane protein assembly and protein translocation across this membrane.

In order to simplify our approaches in elucidating the molecular events underlying these transport processes, we aimed for a single-step method for the purification of the CtxB-Igaβ fusion protein. We describe here the addition of a histidine tag to the C-terminus of CtxB-Igaβ. Histidine tags, which are widely used in various expression systems, allow the efficient affinity chromatography of recombinant products on nickel-NTA-sepharose [8]. We show that a particular glycine-histidine tag interfered neither with correct outer membrane integration of Igaβ nor with the translocation of the passenger, CtxB.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli UT2300, UT5600 (*ompT*) [9] and JK321 (*ompT, dsbA*) (Krämer et al., submitted) were used as host strains for the plasmids pTK59 [6] and pAS02. Transformants were grown at 37° C in LB medium or on LB agar plates, supplemented with ampicillin at a final concentration of 100 μg/ml.

2.2. Construction of plasmid pAS02

Plasmid pTK59 [4] was amplified by PCR using the primers AS07 (GCGTCTAGATAAAATAC-CAAATTCATAGCAAAATA) and AS08 (CGT-CTAGATTAGTGATGGTGATGGTGATGACCGC-CACCGAAACGAATCTGTATTTTAATTTGT). The purified PCR-product was digested with *Xba*I and self-ligated. The sequence of the respective joining region of the new plasmid, pAS02, was determined on a 373A automated DNA sequencer (Applied Biosystems) using the Taq Dye Primer Cycle Sequencing Kit.

2.3. Cell lysates, SDS-PAGE and immunoblotting

Cells were washed once with PBS, resuspended in sample solution, and lysed by boiling for 2 min and extensive sonication with a Branson cell disruptor.

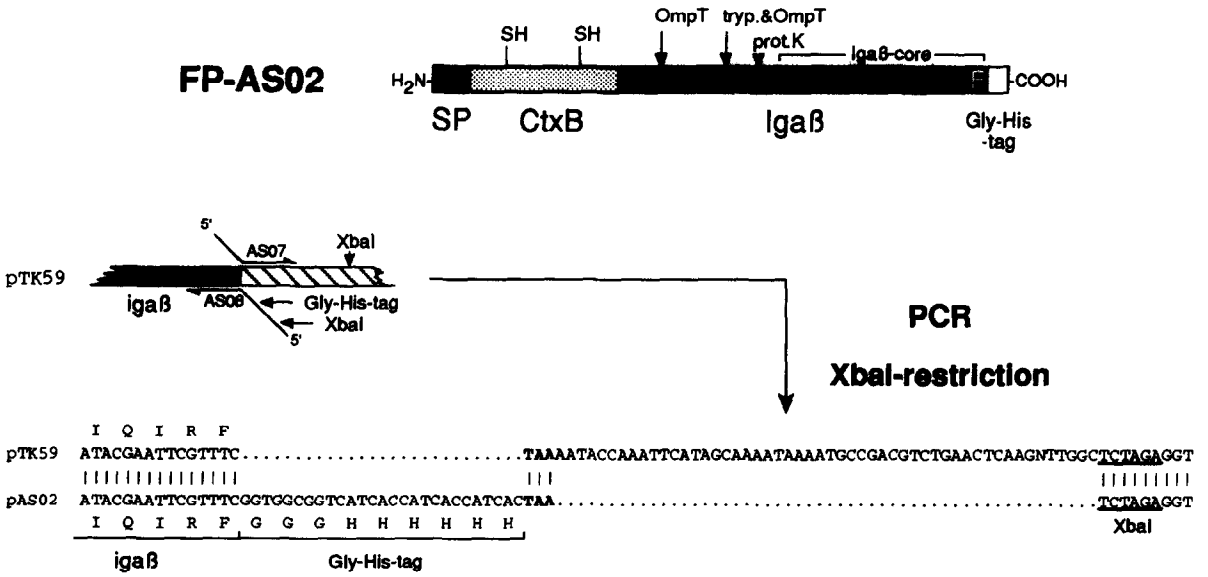

Fig. 1. Schematic representation of fusion protein FP-AS02 and the construction of plasmid pAS02. Approximate locations of relevant features of FP-AS02 are shown. SP, designates the signal peptide of CtxB. The two single cystein residues (-SH) of CtxB are indicated. Arrowheads show cleavage sites of proteinase K (prot. K), trypsin (tryp.) and OmpT protease, all being situated outside the membrane integral part of Igaβ (Igaβ-core). The C-terminal phenylalanine of Igaβ is indicated (F). To illustrate the differences between the parental plasmid pTK59 and pAS02, 3'-regions of *igab* are compared. Dots indicate missing nucleotide positions of either plasmid.

After another 3 min of boiling an aliquot of each sample corresponding to 100–200 μl of liquid culture was analysed by SDS-PAGE and immunoblotting, which were performed as previously described [4] with the modification that the Iga β-specific rabbit anti-FP42 serum was used in 1:170 dilution.

2.4. Protease treatment of intact cells

Transformants were grown in LB-medium supplemented with 5 mM 2-mercaptoethanol. Cells corresponding to 1 ml liquid culture (A_{550} ~ 0.9) were washed twice with PBS, supplemented with 5 mM $MgCl_2$ and resuspended in 200 μl TBS. Samples were incubated for 2 h at 4° C with trypsin or proteinase K at a final concentration of 0.5 mg/ml. Control samples were incubated without proteases. Proteolysis was terminated by the addition of Na-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and phenylmethylsulfonyl fluoride (PMSF) at final concentrations of 135 μM and 1 mM, respectively. Proteins were precipitated with trichloracetic acid at 10% final concentration for 30 min on ice. After centrifugation for 20 min at 13000 rpm the precipitate was washed with ethanol (80% v/v), dried, resuspended in 200 μl sample solution and boiled for 5 min. Sample volumes corresponding to 100–200 μl liquid culture were analysed by SDS-PAGE and immunoblotting.

3. Results

3.1. Construction of a CtxB-Igaβ fusion protein with a C-terminal histidine tag

Plasmid pTK59 encodes the fusion protein FP-TK59, which was previously referred to as B59 [4,5]. It consists of the CtxB subunit with its native signal peptide and the β-domain of the *N. gonorrhoeae* MS11 IgA protease precursor [2]. To obtain plasmid pAS02 which encodes the new CtxB-Igaβ fusion protein FP-AS02 containing three additional glycine and six histidine residues at the C-terminal end, plasmid pTK59 was PCR-amplified using the synthetic oligonucleotide pair AS07/AS08 (Materials and methods). The PCR-product was cleaved with *Xba*I, thus deleting a 52-bp fragment in the non-coding region downstream of *igab*, ligated and transformed into *E. coli* (Fig. 1). Expression of the gene was achieved by the constitutive promoter P_{tk} [4]. *E. coli* clones, harbouring plasmid pAS02, were identified by immunoblotting of cell lysates with the Igaβ-specific serum anti-Fp42. Due to the glycine-histidine tag, fusion protein FP-AS02 had a slightly altered electrophoretic mobility compared to FP-TK59 (Figs. 2–4).

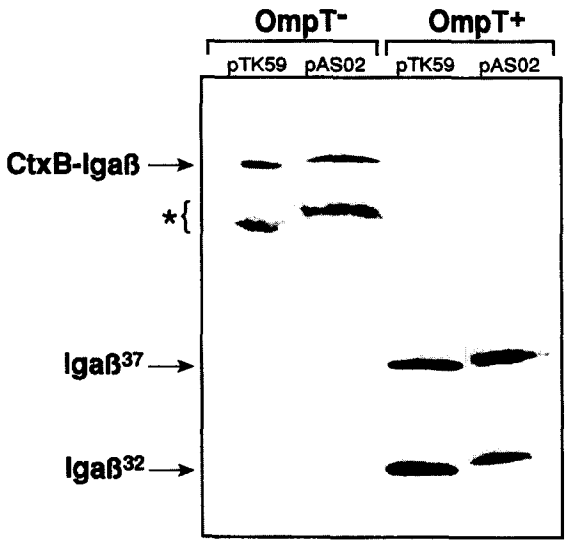

Fig. 2. Processing of FP-AS02 and FP-TK59 by *E. coli* endogenuos OmpT-protease. Immunoblot analysis was performed on cell lysates of an OmpT-negative (UT5600) and an OmpT-positive (UT2300) strain. Arrows specify intact FP-TK59 (CtxB-Igaβ) and specific cleavage products with a molecular weight of 37 kDa (Igaβ37) and 32 kDa (Igaβ32). The asterisk indicates Igaβ protein bands which result from the periplasmic degradation of CtxB not translocated across the outer membrane (see 3.3).

3.2. Outer membrane assembly of fusion protein FP-AS02

Igaβ directs the transport of N-terminally attached passenger proteins across the outer membrane. To this end, the C-terminal portion of Igaβ (the Igaβ-core, see Fig. 1) integrates into the outer membrane and is thought to form a pore that allows the translocation of the passenger protein [3]. To investigate whether FP-AS02 correctly assembles in the outer membrane, protease accessibility studies were performed with intact bacterial cells. In these

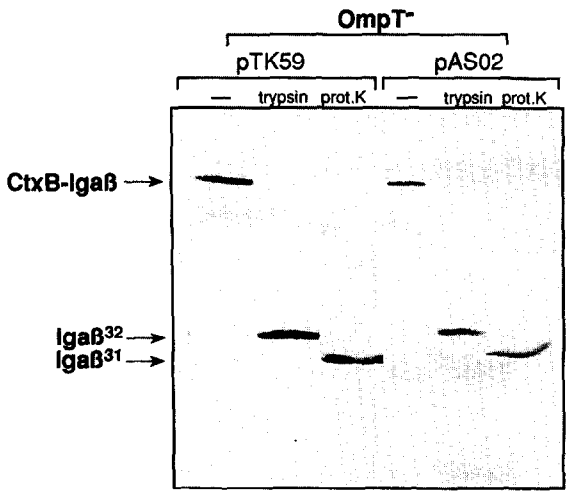

Fig. 3. Accessibility of FP-AS02 and FP-TK59 to externally added proteases. *E. coli* UT5600 (*ompT*) cells grown in reducing media were treated with trypsin and proteinase K and cell lysates compared by immunoblot analysis. Intact fusion protein FP-TK59 (CtxB-Igaβ) encoded by plasmid pTK59 and cleavage products are specified by arrows.

analyses, we made use of the *E. coli* endogenous outer membrane protease OmpT [9] and of externally added trypsin and proteinase K. Fig. 1 illustrates the approximate positions of the cleavage sites for these proteases. Endogenous (Fig. 2) and exogenous (Fig. 3) protease cleavage patterns of FP-AS02 perfectly matched the ones of FP-TK59. As previously shown for FP-TK59 [3–5], these distinct cleavage patterns indicate the correct assembly of Igaβ of FP-AS02 in the outer membrane. Therefore, the C-terminal glycine-histidine tag is compatible with the integration of Igaβ into the outer membrane.

3.3. Passenger protein translocation by fusion protein FP-AS02

To facilitate the analysis of whether the β-domain of FP-AS02, modified with the glycine-histidine tag, was still capable of translocating the passenger protein (CtxB) across the outer membrane, the *ctxB-igab* gene fusion on plasmid pAS02 was expressed in *E. coli* JK321, a DsbA- and OmpT-deficient strain (Krämer et al., submitted). DsbA, a periplasmic enzyme, was shown to catalyse the formation of intramolecular disulfide bonds of several periplasmic and extracellular proteins in Gram-negative bacteria [10–12]. The folded and disulfide bond-stabilized conformation of these extracellular proteins is an

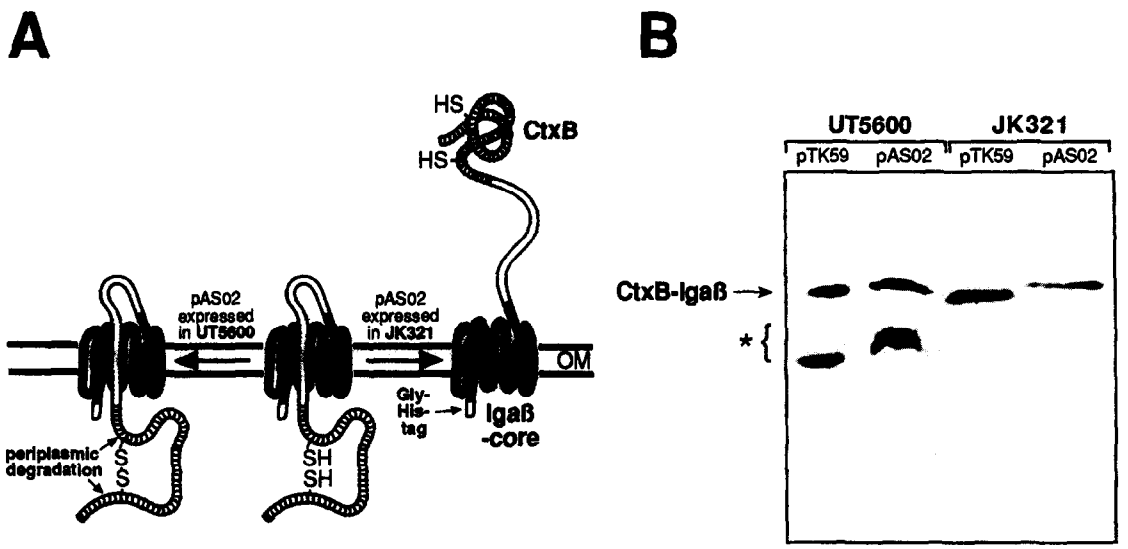

Fig. 4. Passenger protein translocation across the outer membrane by non-tagged (FP-TK59) and tagged (FP-AS02) Igaβ. (**A**) Schematic representaion of the strain-dependent translocation process. In strain JK321 (*dsbA*), the periplasmic disulfide bond formation in CtxB is not catalyzed, thus allowing non-folded CtxB to translocate across the outer membrane. Disulfide bond formation in strain UT5600, grown in non-reducing media, impairs CtxB translocation, which therefore is subjected to degradation by periplasmic proteases. (**B**) Cell lysates of *E. coli* UT5600 (*ompT*) and JK321 (*ompT*, *dsbA*) were compared by immunoblotting. The asterisk indicates periplasmic degradation products, CtxB-Igaβ designates the intact fusion protein.

essential prerequisite for their transport across the outer membrane. In contrast to this, the Igaβ driven extracellular secretion requires a non-folded conformation of the attached passenger protein. If intramolecular disulfide bond formation in CtxB is catalyzed, a substantial proportion of the synthesized CtxB will not be translocated [4]; it remains captured in the periplasm and is thus subjected to degradation by periplasmic proteases [5]. The *dsbA* mutation in strain JK321 abolishes this catalysis and significantly enhances thereby the translocation of CtxB across the outer membrane (Krämer et al., submitted).

When FP-AS02 was expressed in strain JK321 (*dsbA*) no degradation of CtxB was detectable (Fig. 4B). This result strongly suggests that the translocator function of Igaβ in FP-AS02 is not impaired by the glycine-histidine tag. These results were confirmed by analysis of strain UT5600 (pAS02), grown in the presence of 5 mM 2-mercapthoethanol (Fig. 3, see lanes without added proteases), a reducing agent that has been shown to keep CtxB in a reduced, translocation compatible conformation [5]. In contrast, growth of the strains UT5600 (pAS02) and UT5600 (pTK59) in the absence of the agent impaired the translocation of CtxB and thus induced the degradation of CtxB captured in the periplasm. This was indicated by the occurrence of the Igaβ protein band which lacks the CtxB moiety (Figs. 2 and 4B, asterisk).

4. Discussion

The attachment of a glycine-histidine tag to the C-terminal phenylalanine of a hybrid outer membrane protein, the CtxB-Igaβ fusion protein FP-TK59 [4], was successfully achieved and the influence on (i) the assembly in the outer membrane and (ii) the translocation of the CtxB portion across this membrane was investigated. Recent studies with FP-TK59 and derivatives led to the characterization of the structural requirements of both the Igaβ translocator and passenger protein for successful translocation across the outer membrane ([1,3–5], Krämer et al., submitted). These studies provided a basis for the interpretation of endogenous and exogenous protease cleavage patterns of Igaβ fusions and thus allow to judge the features of FP-AS02 in comparison with FP-TK59. Since no significant differences between the two proteins are evident (Figs. 2–4) the glycine-histidine tag clearly does not affect the function of Igaβ in FP-AS02.

We also tried to introduce slightly varying histidine tags at the C-terminal end of FP-TK59 by the same strategy we employed for FP-AS02 (data not shown). However, we failed to receive any transformants with a histidine tag attached directly, or via a Gly-Glu-Gly-Glu amino acid spacer to the C-terminal phenylalanine. This suggests a toxic effect of these tags due to an incompatibility with folding of Igaβ, or its proper position in the outer membrane. The C-terminal phenylalanine seems to be a common feature of nearly all bacterial outer membrane proteins [13] and, with its aromatic ring in close contact to the hydrophobic core of the outer membrane, is supposed to play an important role both in the outer membrane assembly [13,14] and the maintenance of outer membrane protein function [6,15]. C-terminal modifications of outer membrane proteins are therefore likely to affect adversely outer membrane insertion and assembly processes [13,14]. The sequence Gly-Gln-Gly-Glu was derived from the phenylalanine-attached C-terminal periplasmic tail of the OmpA protein [16]. That this naturally occurring sequence, in connection with a histidine tag, could not be fused to the C-terminus of Igaβ indicates that individual solutions must be found regarding C-terminal extensions for any outer membrane protein of interest. However, our study demonstrates that such extensions are possible without structural or functional implications for the modified protein.

Thus, fusion protein FP-AS02 represents a new, suitable tool in studies directed towards a further understanding of the molecular processes during outer membrane assembly and translocation. It is advantageous over FP-TK59 in that it can be purified on nickel-NTA-sepharose from crude cell lysates in a single step (data not shown). The new fusion protein FP-AS02 is therefore useful in order to facilitate in vitro reconstitution and translocation experiments, where highly purified protein is required, as well as for the large-scale preparation required for crystallization purposes. Furthermore, it allows to selectively precipitate transport intermediates in in vivo pulse chase experiments (data not shown) in which the time-course of Igaβ driven translocation may be

followed. A tempting idea is to make use of the glycine-histidine tag to enrich accessory molecules that possibly are involved in protein secretion and folding. Such cross-linking experiments might shed light on still unresolved questions concerning the existence of general periplasmic chaperones [17] for review, that are suggested to participate in folding and de-folding processes and in membrane insertion of outer membrane proteins.

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