Biochemical and Biophysical Research Communications 394 (2010) 962–965

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The tip region of the MacA α-hairpin is important for the binding to TolC to the *Escherichia coli* MacAB–TolC pump

Yongbin Xu [a,1], Se-Hoon Sim [b,1], Saemee Song [b], Shunfu Piao [a], Hong-Man Kim [b], Xiao Ling Jin [a], Kangseok Lee [b,*], Nam-Chul Ha [a,**]

[a] Department of Manufacturing Pharmacy, College of Pharmacy and Research Institute for Drug Development, Pusan National University, Busan 609-735, Republic of Korea
[b] Department of Life Science (BK21 Program), Research Center for Biomolecules and Biosystems, Chung-Ang University, Seoul 156-756, Republic of Korea

ARTICLE INFO

Article history:
Received 9 March 2010
Available online 20 March 2010

Keywords:
Multidrug resistance
MacA
TolC
Assembly model

ABSTRACT

The tripartite efflux pump MacAB–TolC found in Gram-negative bacteria is involved in resistance to antibiotics. We previously reported the funnel-like hexameric structure of the adaptor protein MacA to be physiologically relevant. In this study, we investigated the role of the tip region of its α-hairpin, which forms a cogwheel structure in the funnel-like shape of the MacA hexamer. Mutational and biochemical analyses revealed that the conserved residues located at the tip region of the α-hairpin of MacA play an essential role in the binding of TolC. Our findings offer a molecular basis for understanding the drug resistance of pathogenic bacteria.

1. Introduction

The tripartite efflux pumps of Gram-negative bacteria have been implicated in the intrinsic drug resistance and toxin secretion of pathogenic bacteria [1–4]. Diverse xenobiotic or protein toxins are selectively pumped out to the external medium depending on the tripartite efflux pumps [2,5]. These pumps typically consist of three essential components. An inner membrane transporter (IMT) provides energy for the transport of substrates [6] and an outer membrane factor (OMF) connects with IMT in the periplasm, providing a continuous conduit to the external medium [6,7]. The third essential component is an adapter protein called a membrane fusion protein (MFP) that connects the IMT to the OMF in the periplasm [5]. The crystal structure of MFPs reveals them to consist of a membrane proximal domain, β-barrel domain, lipoyl domain, and α-hairpin domain [8–12]. Structural and functional results suggest that the α-hairpin domain of MFP is involved in interactions with TolC [8,13].

MacAB–TolC, identified as a macrolide-specific extrusion pump [14], has also been implicated in enterotoxin secretion by *Escherichia coli* [15]. IMT-MacB is an ABC transporter that uses ATP hydrolysis as its driving force [14] and is characterized as a homodimeric protein [16]. MFP-MacA connects MacB to TolC and shares a high sequence similarity with AcrA and MexA, both of which are associated with the resistance nodulation cell division-type transporters that are structurally distinct from MacB [5].

In our previous work, we suggested that the funnel-like hexameric structure of MacA is physiologically relevant using structural, biochemical, and genetic approaches [9]. The crystal structure of MacA suggest a 'MacA-bridging model' in which MacA directly contacts TolC in a tip-to-tip manner intervening between TolC and MacB [9,17]. In this study, we investigate the role of the tip region of the α-hairpin of MacA in support of the idea of tip-to-tip binding between MacA and TolC.

2. Materials and methods

2.1. Strains and plasmids

The construction of the *acrAB* deleted *E. coli* strain BW25113, Δ*acrAB*, has been previously described [9]. To construct pMacAB1-R131A, -L135D, -S142D, and R131A/L135D, DNA fragments encoding mutant MacA proteins were created using the overlap extension PCR method. The resulting PCR DNA was digested with EcoRI and HpaI and ligated into the same sites in pMacAB1. The outside primers used were MacA-F (5′-ATCATATGAAAAAGCGGAAAACCGT) and MacAY275A-R (5′-GAAAATAGCGTCGTTAACCT). The mutagenic primers used were MacA-R131A-R (5′-ACGTTGCTG**CGC**GGAATACGTCACCCGCGCCA; mutated nucleotides in bold) and MacA-R131A-F (5′-ACGTATTCC**GCG**CAGCAACGTCTGGCACAAACG; mutated nucleotides in bold) for MacA-R131A, MacA-L135D-R (5′-**ATC**ACGTTGCTGACGGGAATACG; mutated nucleotides in bold) and MacA-L135D-F (5′-TTCCCGTCAGCAACGT**GAT**GCACAAACGAA

* Corresponding author.
** Corresponding author.
E-mail addresses: kangseok@cau.ac.kr (K. Lee), hnc@pusan.ac.kr (N.-C. Ha).
[1] These authors equally contributed to this work.

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.03.097

GGCT; mutated nucleotides in bold) for MacA-L135D, MacA-R131A-R and MacA-R131A-L135D-F (5′-CGTATTCC**GCG**CAGCAACGT**GAT**GCACAAACGAAGGCTG; mutated nucleotides in bold) for MacA-R131A/L135D, and MacA-S142D-R (5′-GCTG**GTC**AACAGCCTTCGTTTGTGC; mutated nucleotides in bold), MacA-S142D-F (AACGAAGGCTGTT**GAC**CAGCAGGATCTCGACA; mutated nucleotides in bold) for MacA-S142D.

2.2. Over-expression and purification of recombinant proteins

The expression and purification of *Actinobacillus actinomycetemcomitans* MacA (Aa MacA) and *E. coli* MacA have been previously described [9,18]. The substitution mutants Aa MacA and *E. coli* MacA proteins were generated by the QuikChange method (Invitrogen).

2.3. In vitro binding assay

The recombinant proteins of hexahistidine-tagged Aa MacA (wild type and mutants) and the GST-fused Aa TolC periplasmic region were expressed and purified as previously described [17]. Ten microliters of glutathione resin, bound to the GST-fused Aa TolC protein or resin alone, were incubated with Aa MacA (wild type or mutants) at 4 °C for 1 h. The resin was washed with 20 mM Tris–HCl (pH 8.0) buffer containing 150 mM NaCl and 2 mM 2-mercaptoethanol, and it was then eluted with the same buffer supplemented with 20 mM glutathione. The eluted fractions were analyzed by SDS–PAGE, and the protein bands were stained with Coomassie blue.

2.4. Measurement of minimum inhibitory concentration (MIC)

The measurement of MIC has been previously described [9].

3. Results and discussion

3.1. The conserved residues of the MacA α-hairpin tip region are functionally important

In the funnel-like hexameric structure of MacA, the end of the funnel mouth exhibits a cogwheel-shaped α-barrel composed of six α-hairpin tip regions (Fig. 1A) [9]. This six-membered cogwheel of the α-barrel suggests that the α-hairpin tip region may pertain to the binding to TolC (Fig. 1A) [9]. To examine the functional importance of the α-hairpin tip region of MacA, three of the strictly conserved residues found in all subfamilies of MFPs (Arg131, Leu135, and Ser142 in the *E. coli* MacA) (Fig. 1) were mutated, and the resulting MacA mutants were tested for their ability to form a functional drug efflux pump. For this experiment, an *E. coli* strain with deleted *acrAB* genes (BW25113Δ*acrAB*) was used, as the function of MacAB–TolC can be easily measured in the absence of the major multidrug resistance gene *acrAB* [14,19,20]. As

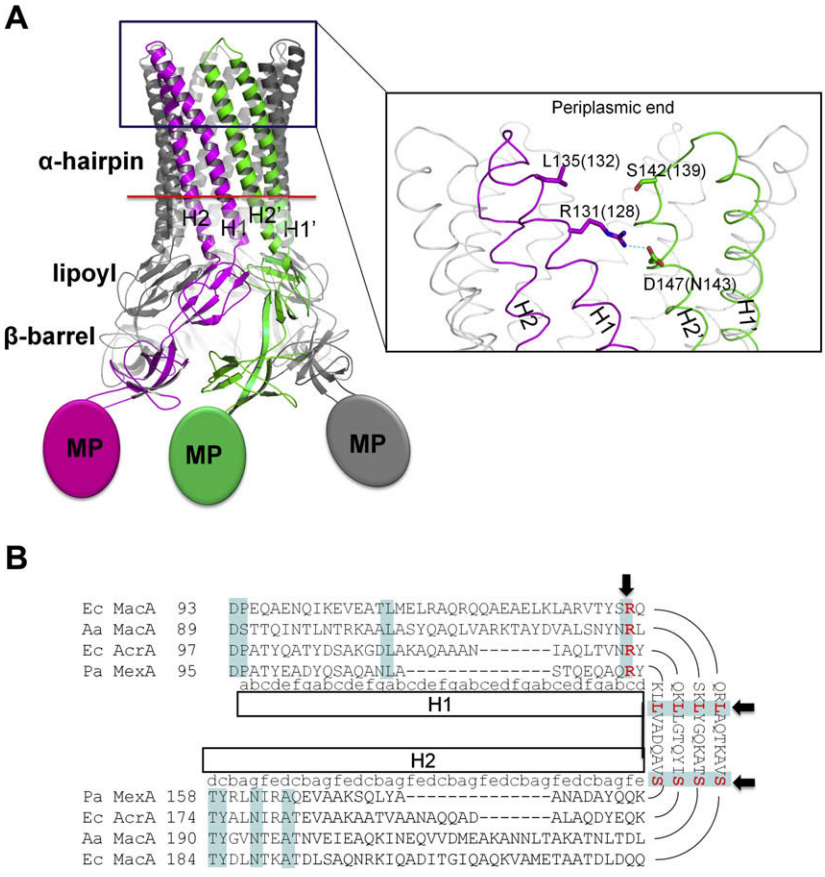

Fig. 1. The structural features and importance of the α-hairpin tip region of MFPs. (A) Ribbon representation of the *E. coli* MacA hexameric structure and a close-up view of the α-hairpin tip region. Four domains are labeled, and the membrane proximal (MP) domains are shown schematically. The two protomers, colored in magenta and green, represent the asymmetric unit in the crystal (PDB code 3FPP) [9], and each α-helix are labeled (H1, H2, H1′, and H2′). The three strictly conserved residues are shown in the stick representations with labels in a format of *E. coli* MacA number (*A. actinomycetemcomitans* MacA number). (B) Sequence alignment of α-hairpins from four MFPs. The corresponding heptad position is shown below or above the sequence. The strictly conserved residues are shaded, and particularly strictly conserved residues at the tip region are indicated by arrows. Note that the differences in the length of α-hairpins are in multiples of seven. In the sequence alignment, Ec stands for *E. coli*, Aa for *A. actinomycetemcomitans*, and Pa for *P. aeruginosa*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)