行政院國家科學委員會專題研究計畫成果報告

國科會專題研究計畫成果報告撰寫格式說明

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一、中文摘要

GspG, -H, -I, -J, -K 蛋白又稱為假纖毛蛋白，參於葛蘭氏陰性菌 Type II 蛋白分泌機轉，推測它們在分泌機器中會形成纖毛結構以幫助蛋白質分泌，但該纖毛結構至目前為止仍無直接證據證明它的存在。在本研究中，我們以膠質篩濾層析法、蔗糖密度梯度離心、親和性管柱層析法間接證明 GspG 在內外膜會形成一類似纖毛的結構，這個發現對於了解 GspG 在蛋白分泌機器中所扮演的腳色有很大的幫助
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關鍵詞：專題計畫、報告格式、國科會

Abstract

GspG, -H, -I, -J, -K proteins are members of pseudopilin family that are required for the type II secretion pathway in the step for translocating extracellular proteins across the outer membrane in Gram negative bacteria. They were proposed to form pilus-like structure but demonstrated to be so only in the case of PulG of *Klebsiella oxytoca* with recent electron microscopic data. In this study we performed biochemical analysis of the XpsG protein of *Xanthomonas campestris* pv. campestris and detected it as pillar-like structure spanning between cytoplasmic and outer membrane. Subcellular fractionation followed by immunoblot analysis revealed, in addition to the membrane form (MF), a soluble form (SF) of the XpsG. Chromatographic analysis of SF XpsG in absence of detergent indicated that it exists in molecules with sizes larger than 440 kDa. *In vitro* studies indicate that XpsG is prone to aggregate in absence of detergent. In our search for non-functional mutant with minor variation in sequence however defective in forming the large-sized molecules, a mutant XpsG-F120 was obtained. It was neither functional nor dominant negative. It was also no longer detectable in soluble fraction. Moreover, unlike the wild type XpsG, which was distributed in both cytoplasmic membrane and outer membrane upon sucrose gradient sedimentation, it appeared only in the cytoplasmic membrane. Co-expression of wild type XpsG with $(His)_6$-tagged XpsH, but not untagged ones, made SF XpsG bound to nickel and co-eluted with XpsH, suggesting presence of other pseudopilin components in the XpsG-containing large-sized molecules.

Keywords: type II secretion pathway, pseudopili, pillar-like structure

二、緣由與目的

In the type II secretion apparatus that is involved in transport of extracellular proteins across the outer membrane in Gram-negative bacteria, five type IV pilin-like proteins (GspG, -H, -I, -J and -K) have been identified. They were designated pseudopilins [1] for their sequence similarity at the N-terminus with type IV prepilin. The sequence G↓(F/M)XXXE followed by a hydrophobic region constituted of 15-20 amino acid residues is conserved in all pseudopilins. Moreover, the cleavage between G↓F by the type IV prepilin leader peptidase is required for their normal functioning [2-4].

Type IV pilin forms helical polymer (designated as type IV pilus) of 6 nm in diameter and up to several micrometers in length on the surface of a variety of bacterial species. Crystallographic structure of

Neisseria gonorrhoeae pilin revealed an elongated ladle-shaped structure [5]. The N-terminal segment of a long α-helical spine forms the ladle handle. A computational model of a right-handed fiber with five subunits per turn was proposed. The fiber is formed with a core of packed hydrophobic α-helices surrounded by a tubular β-wrap. In the detergent-solubilized state a dumb-bell-shaped dimer is thermodynamically favored for minimal exposure of its N-terminal hydrophobic α-helix.

Similarity of pseudopilins at their N-terminal amino acid sequence with pilin has intrigued the postulation of pilus-like structure formation by pseudopilins [6, 7]. The pilus-like structure was proposed to connect the cytoplasmic membrane with the outer surface of the cell, either for conducting protein secretion through outer membrane [8], or acting as piston to push secreted proteins through the secretion pore located in the outer membrane [9]. The major pilin-like protein PulG of *Klebsiella oxytoca* was shown to form multimers of two to five subunits upon formaldehyde cross-linking [10]. In addition to homodimer formation, XcpT (GspG of *P. aeruginosa*) was also shown to form heterodimers with other pseudopilins as suggested by cross-linking and affinity chromatography [11]. Despite these observations, demonstration of pilus-like structure composed of pseudopilin has been unsuccessful until recently. Using electron microscopy, Sauvonnet et al. [12] observed pilus-like bundles with diameter of 15-25 nm on surface of *Escherichia coli* that overexpressed the *pul* operon of *K. oxytoca*. Presence of PulG in the pilus-like structure was confirmed with immunogold labeling. However, such surface-exposed pilus-like structure could not be detected when the Pul components were expressed at low level. Furthermore, characteristics of the pilus-like structure inside the cells remain unclear.

XpsG, constituted of 143 amino acid residues, is the major pilin-like protein in *Xanthomonas campestris* pv. campestris. We observed that the XpsG of the wild type strain existed in two forms, a major membrane form and a minor soluble form. The XpsG in soluble form was shown to have large sizes upon size exclusion chromatography in absence of detergent. To exclude the possibility that the large-sized molecules in wild type strain may have been formed as un-ordered structures during *in vitro* manipulation, we looked for mutant XpsG that lost its function without affecting protein stability. A mutant XpsG-F120 was obtained. It became undetectable in outer membrane or in soluble fraction. We hypothesize that, as a result of cell passage through French press, the pilus-like structure connecting two membranes in intact cells was broken and released as soluble fraction in wild type strain.

三、結果與討論

Detection of soluble form of XpsG

To characterize the pilin-like protein XpsG, we raised antibody against a thioredoxin-XpsG fusion protein produced in *E. coli*. Upon immunoblot analysis, we detected a distinct band in the parental strain of *X. campestris* pv. campestris XC1701 but absent in the Δ*xpsG* strain XC1713. Molecular size of this protein agrees with the predicted molecular weight of the XpsG protein (15,150 Dalton), indicating that the 16 kDa band appearing on SDS-PAGE represents the XpsG protein. To determine the subcellular location of the XpsG protein, the French press-broken cells were separated into two fractions by ultracentrifugation. While the α-subunit of RNA polymerase was detected only in supernatant, the outer membrane protein XpsD appeared only in pellet. In contrast, the XpsG protein was detectable in both fractions. The proportion of the XpsG protein detected in supernatant is approximately one-fourth of that in pellet. Because hardly any of the membrane protein XpsD could be detected in supernatant, the XpsG protein appearing in supernatant is unlikely to be contaminant as un-precipitated membrane vesicles. We designate it the soluble form (abbreviated as SF) and the XpsG in pellet the membrane form (abbreviated as MF). Theoretically, while the

soluble fraction includes both cytoplasmic and periplasmic fractions, the membrane fraction comprises outer membrane and cytoplasmic membrane.

Chromatographic analysis of MF XpsG and SF XpsG

To determine if the XpsG protein forms pilus-like structure inside the cell, we attempted its purification by conducting ion exchange chromatography followed by gel filtration. Upon ion exchange chromatography, the Triton X-100-extracted MF XpsG fractionated at 7-11 min. Further analysis on Superdex HR200 size exclusion column equilibrated with 1% Triton X-100 revealed that the MF XpsG fractionated with a major peak at 22-26 min ranging in molecular size from 66-443 kDa, which appears to be too small for pilus-like structure. In addition, when the column was equilibrated with 0.5% deoxycholate (DOC), the MF XpsG appeared at 29-32 min with molecular size of approximately 32 kDa, twice the size of XpsG, suggesting that the XpsG-containing multimeric structure is likely to be disrupted upon detergent treatment. Therefore it not feasible to purify intact pilus-like structure, if exist, from the membrane fraction. On the other hand, the SF XpsG behaved differently. In absence of any detergent, it fractionated at 11-13 min upon ion exchange chromatography. Analyzed on size exclusion column equilibrated with buffer without detergent, the SF XpsG fractionated in near void volume, implying its nature as being large-sized molecules with sizes greater than 443 kDa. The assembled pseudopilin, unlikely to remain in the cytoplasm, probably has crossed cytoplasmic membrane. Assuming that the XpsG protein forms pillar-like structure in periplasm with one end buried in cytoplasmic membrane and the other in outer membrane, one could envision that the part exposed to the periplasmic space may easily be broken from their membrane anchorage during cell disruption. We postulate this be the source of the SF XpsG.

Effect of detergent on SF

XpsG Size exclusion chromatography of SF XpsG in presence of Triton X-100 or DOC resulted in the same elution profiles as those of MF XpsG (data not shown), implying that the former may share similar characteristics, and possibly similar overall structure, as those of the latter. By varying concentrations of DOC in the equilibration buffer of size exclusion column, we observed appearance of the SF XpsG as forms other than large-sized molecules eluting at near void volume or dimer-sized molecules eluting at 30-31 min. A wide distribution of XpsG (between 16-29 min) was clearly detected at 0.1% DOC. Apparently, molecular sizes of the SF XpsG vary significantly with the concentrations of DOC. In an attempt to re-assemble the large-sized XpsG from dimeric XpsG, we collected the 0.5% DOC-dissociated XpsG and applied on a second size exclusion column in absence of DOC. The dimer-sized XpsG was no longer detectable. Neither did it resume its original large size fractionating at near void volume. Instead, the XpsG appeared at 19-27 min, suggesting that some unknown factor required for reassembly may have been removed. It is concluded that size determination of the XpsG multimeric structure in presence of detergent is not reliable. Moreover, the results imply that the XpsG is prone to assemble or aggregate in absence of detergent. The detected large-sized XpsG in soluble form, instead of representing the pilus-like structure, may have arisen as an artifact from in vitro process. In order to find out the biological relevance of the large-sized molecules, we tried searching for XpsG mutant with only minor changes in protein sequence, however, has lost its biological function and large-sized form concomitantly.

Analysis of XpsG-F120 mutant protein

In the search of nonfunctional XpsG mutant whose protein amount remains unaffected, we obtained one with insertion of seven amino acid residues DH_5V downstream of F120 (encoded by the plasmid pF120). Overexpression of the mutant protein XpsG-F120 did not exert negative dominance effect in the parental strain XC1701, neither did it complement the Δ*xpsG* strain XC1713. Upon SDS-PAGE, the XpsG-F120 migrated

slower than the wild type XpsG protein, reflecting its increase in molecular weight (16,050 Dalton). An extra band migrating faster than the wild type XpsG was detected only in the membrane fraction whenever the XpsG-F120 was expressed. It is probably a degradation product of the XpsG-F120 protein generated during preparation. Subcellular fractionation analysis of the XpsG-F120 expressed in XC1713 revealed that the mutant protein was only detectable in the membrane form. In contrast, the wild type XpsG protein that co-expressed with the mutant XpsG-F120 protein in XC1701/pF120 remained in two forms. Interestingly, the XpsG-F120, although expressed to a substantial amount, did not mingle with the wild type XpsG protein in formation of the large-sized molecules in SF.

To examine if the MF XpsG from wild type and mutant strains possess the same characteristics, we analyzed the distribution of XpsG protein between two membrane bilayers. Sucrose gradient sedimentation analysis revealed that the wild type XpsG co-fractionated with both the cytoplasmic membrane marker succinate dehydrogenase (data not shown) in fractions 11-17 and the outer membrane marker OprF (data not shown) in fractions 22-28. This observation agrees with the hypothesis that the assembled XpsG forms pillar-like structure across periplasm and attaches with both outer and cytoplasmic membranes *in vivo*. In contrast, the mutant XpsG-F120 only appeared in the cytoplasmic membrane fractions. The absence of mutant protein in both soluble and outer membrane fractions suggests that the XpsG-F120 has lost its ability to form the pillar-like structure, therefore accumulates only in cytoplasmic membrane.

Chromatographic analysis of extracellular XpsG

By suspending cells harvested from plate, Sauvonnet et al [12] observed detachment of PulG-containing pilus-like structure from surface of the cells that overexpress the Pul secreton components. This prompted us to inspect if any large-sized molecules could be detected in extracellular fraction. We observed that detection of XpsG in extracellular fraction was possible only when XpsG was overproduced to a significantly higher level than that in wild type strain. Secretion remains normal in this complemented strain XC1713/pFG (data not shown). Further analysis upon size exclusion chromatography in absence of detergent indicated that the extracellular XpsG of XC1713/pFG has similar elution profile as that of the SF XpsG from XC1701.

Co-fractionation of XpsG with another pseudopilin component upon Ni-affinity chromatography

Heterodimers of XcpT with XcpU, -V and -W, the pseudopilins of *P. aeruginosa*, have been demonstrated with cross-linking studies [11]. To examine if other pseudopilins are present in the XpsG-containing large-sized molecules, we made use of the nickel-binding property of $(His)_6$-tagged XpsH protein and analyzed the co-expressed wild type XpsG protein upon affinity column chromatography followed by immunoblot analysis of XpsG. $(His)_6$-tagged XpsH protein was shown to complement its chromosomal mutant strain XC1717 (unpublished result). It shows that in XC1717 the XpsG was co-eluted if the $(His)_6$-tagged XpsH (encoded by pFH-His), but not the wild type XpsH (encoded by pFH), was expressed. The elution profile of XpsH was confirmed with antibody against XpsH. Conversely, the XpsH was demonstrated to be co-fractionated only with the $(His)_6$-tagged XpsG. Failure in detection of the XpsH protein in the crude extract may due to the low expression of the chromosomal gene. These results demonstrate that the SF XpsG contains at least another pseudopilin component, although may not be equal in their molecule number.

五、參考文獻

[1] 上述成果已發表於 *Biochem. J.* **365**, 205~211, (2002) Hu, N.-T., Leu, W.-M., Lee, M.-S., Chen, A., Chen, S.-C., Song, Y.-L., and **Chen, L.-Y.** XpsG, the major pseudopilin in *Xanthomonas campestris* pv. campestris,forms a pilus-like

structure between cytoplasmic and outer membranes.

[2] 行政院國家科學委員會，*專題研究計畫成果報告編寫須知*，民國八十二年十一月。

附件：封面格式

行政院國家科學委員會補助專題研究計畫成果報告

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※ 十字花科黑腐病菌類似纖毛 XpsGHIJ 蛋白的結構與功能關係研 ※

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共同主持人：

計畫參與人員：

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