行政院國家科學委員會專題研究計畫 成果報告

探討 CadA 鎘運輸蛋白上的功能區域
研究成果報告(精簡版)

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計畫參與人員：助教級-兼任助理：林小芸

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中 華 民 國 96 年 10 月 30 日

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

探討CadA 鎘運輸蛋白上的功能區域

Characterization of the function domains of CadA Cd^{2+}-ATPase

計畫類別：■ 個別型計畫　　□ 整合型計畫

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成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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執行單位：中山醫學大學醫學檢驗暨生物技術學系

中　華　民　國　　96　　年　　8　　月　　30　　日

一、中英文摘要

金黃色葡萄球菌所攜帶的抗鎘的CadA蛋白，與大腸桿菌抗銅的CopA蛋白，都屬於P-type ATPase中的CPx-type ATPase。這類運輸金屬的膜蛋白，具有一些獨特的序列及結構上的特徵，像是在蛋白的N端上皆具有Cys-X-X-Cys序列，並且在第六個穿膜區域，具有Cys-Pro-X序列。本研究的目的，就是以直接的生化方法，探討這些CPx-type ATPase結構上所特有的區域，及其所扮演的功能性角色，同時這類的蛋白皆具備有相當程度的相同胺基酸序列，而CPx-type ATPase如何辨識不同的金屬離子，並達成其功能，這是對於進一步認識CPx-type ATPase所必須的資訊。在本研究中，利用雜交蛋白的方法，將CadA及CopA兩種功能迥異的CPx-type ATPases，進行部分區域置換的雜交蛋白，分別製造CadA/CopA、以及CopA/CadA的雜交蛋白。並利用這些雜交蛋白分別進行抗鎘、抗銅的分析。研究結果發現，CXXC對於CopA蛋白可能不具篩選特異性受質的角色，而受質特異性的決定位置，可能在CopA蛋白的其他位置。然而，對於CadA蛋白，CXXC在受質特異性方面，可能具備某些重要性的角色，而CXXC以及所在的N端蛋白區域，也可能是扮演調節性角色，對於執行CadA功能相當重要。

關鍵詞：雜交蛋白、CadA、CopA、CPx-type ATPase

Abstract

Staphylococcal CadA and *E. coli* CopA are both P-type cation-transporting ATPases confer resistances to cadmium and copper, respectively. These proteins have been further categorized into a CPx-type ATPase family, which are characterized by some unique sequence features including the 1-6 N-terminal Cys-X-X-Cys repeats and the sixth transmembrane-buried Cys-Pro-X motif. However, how these proteins identify a variety of substrates and roles of conserved domains have yet to be determined. In this study, a chimera protein strategy was taken to create hybrid proteins with either N/C-terminal portion of CadA fused with C/N-terminal portion of CopA. The clones with these hybrid proteins were assayed their resistances. Our results have shown that CXXC might play different roles in CadA and CopA. In CopA, the CXXC alone does not determine its substrate-specificity. And the copper-specificity of CopA might rely on other locations of the protein, however the CXXC play a supporting role for the function. On the other hand, the CXXC motif in CadA is more important for its substrate-specificity and alone with the N-terminal domain of CadA play a regulatory role in CadA activity.

Key words: Chimera proteins, CadA, CopA, CPx-type ATPase

二、報告內容

前言

Staphylococcal plasmid pI258 carries *cadCA* operon for full resistance to cadmium [1]. Two genes are found in this cadmium resistant operon, called *cadA* and *cadC* [2]. The CadC was previously believed to be a specific cadmium binding protein, however, later data have shown the CadC is a negative regulator for *cadA* gene expression and released from its function in the presence of certain cations [3]. Studies have also shown that CadA is responsible for the cadmium resistant phenotype through an ATP driven pump [4]. Amino acid analysis of CadA protein have shown that CadA is a P-type ATPase [2], and later re-classified into as P_1-type ATPases, due to some unique structural features to other ATPases [5].

The P_1-type ATPases transport certain heavy metals across cell membrane energized by the hydrolysis of ATP [5]. Even though general believe that P_1-type ATPases transport cation (more specifically, the heavy-metals), however only few were demonstrated their transport activities biochemically, including staphylococcal CadA transports Cd^{2+} ion [4]. All these P_1-type ATPases contain eight transmembrane segments, not like other P-type ATPases which proteins trans-across the membrane six to ten times [6]. Some unique features are found in all this class of ATPases including one to six repeated intracellular N-terminal conserved GXTCXXC motif(s), the possible initial binding site for heavy metals [5,6]. Learning from CopA, a Cu^{2+}-transporting ATPase, and alone with another two P_1-type ATPases, the Wilson and Menkes diseases proteins, have demonstrated that some of these motif(s) binds copper *in vivo* and *in vitro*, if not all [7]. Another unique structural feature was found in this class of ATPases is the CPx motif, located within the 6th transmembrane segment (TM) of the proteins and believed to form an ion transduction domain or ion channel within the membrane [5].

No crystalline structure is available for CadA and other P_1-type ATPases so far. However, based on the hydropathy predictions of all P_1-type proteins, a two-dimensional topology model was proposed [5]. Not until recently, the membrane topology of P_1-type ATPases was studied biochemically from other lab and ours [6,8,9], and the data shown that a CadA homologous from Gram-negative *Helicobacter pylori* have shown eight TMs [9], same to the Staphylococcal CadA is an eight-TM protein [6]. However, even the topography of CadA is solved; the catalytic characterizations of this class of enzymes are still far beyond to be understood. For example, how these P_1-type ATPases identify their substrates specifically even when they all shared a high sequence homology. Furthermore, how metal ions are handled by P_1-type ATPases when ions are passing through the membranes.

In this study, we took the advantage of using two much well-characterized P_1-type ATPases, the Staphylococcal CadA and *E. coli* CopA, which recognize different substrates, e.g. CadA recognizes and transports cadmium, CopA's substrate is copper, and used them as models to first disclose how the substrates are recognized by different P_1-type ATPases.

研究目的

In this research, we decide to disclose the structural features of P_1-type ATPases using some currently well-studied P_1-type ATPases, the Staphylococcal CadA and *E. coli* CopA, to understand the functional roles of those unique conserved domains in this class of enzymes. Most of our efforts is planning to characterize the N-terminal CXXC motif, and to understand how this motif is affected the enzyme activity. Also, why evolution decided P_1-type ATPases need only one N-terminal CXXC motif to confer resistance to cadmium, as found in Staphylococcal CadA[2], however two N-terminal CXXC motifs

are acquired for copper resistance in *E. coli* CopA [7], and six are found in either Wilson's protein or Menkes protein [8], even the latter two proteins share with the same substrate as the *E. coli* CopA. Therefore, it will be very important to understand how substrates are recognized by these P_1-type ATPases, as the previous wisdom said "the N-terminal CXXCs is/are responsible for the metal initiate binding" [5,6]. Other domains or amino acid residues are definitely involved in this substrate-binding decision. A throughout searching for the amino acid is not possible, however a chimera strategy of using two different P_1-type ATPases with different substrate-specificities will be possible to answer the question as we have proposed in this research.

文獻探討

So far there are no clear answer to tell what is the role of the N-terminal CXXC motif in P_1-type ATPases. Efforts have been made in the past to solve the problem, including the studies of CadA in *Listeria monocytogenes*, a homolog of Staphylococcal CadA. In their *in vitro* transcription/translation experiments, they found even without the N-terminal CXXC, the CadA still poses the ATPase activity, Cd^{2+}-transporting activity, and formation of phosphorylated intermediate [11,14]. It was suggested that the N-terminal CXXC might be a ion-sensor to regulate the transporting activity [11]. However, different conclusions have been made from the observation of Menkes and Wilson's proteins where they found their six N-terminal CXXC motifs showed different affinities to different cations and suggesting the role for N-terminal CXXC is for direct metal binding [8]. Studies on ZntA [10], another P_1-type ATPases, showed resistance when absence of its N-terminal CXXC motif [12]. Furthermore, N-terminal CXXC deleted ZntA remain its ATPase activity in the presence of different metal ions, suggesting N-terminal CXXC does not play a role in substrate determination [12]. However, Fan *et al* have demonstrated that N-terminal CXXC is important for the copper resistance in *E. coli* CopA [13]. When removed, the copper resistance lost [13].

Based on these observations from different studies, we know that we are still far from understanding the role of this unique sequence of P_1-type ATPases, the N-terminal CXXC motif. So far, four speculations have been proposed for the CXXC motif(s); there are: 1) Substrate specificity; 2) A metal sensor; 3) Initiate binding to metal ion which will be subsequently transport [15]; and 4) A metal chaperon [16]. In our study, we are using chimera strategy to fuse portions of two different P_1-type ATPases to disclose the role of the CXXC motif, as well as the role of the buried 6^{th} transmembrane CPx motif to fully understand how P_1-type ATPases decide and transfer their substrates.

研究方法

In this study, we are going to take the chimera approach to identify the possible role of those conserved domains of P_1-type ATPases and therefore some chimera proteins will be prepared, including chimera proteins with CadA/CopA (AP clone; N-terminal portion of CadA fused with C-terminal portion of CopA) and CopA/CadA (PA clone;N-terminal portion of CopA fused with C-terminal portion of CadA). These chimera proteins will be determine their secondary structures and their resistance activity to either cadmium (substrate for CadA) or copper (substrate for CopA) will be determined.

結果與討論（含結論與建議）

In order to understand the role of N-terminal CXXC in P_1-type ATPases, chimera proteins were prepared from two P_1-type ATPases with different substrates, the Staphylococcal CadA transport Cd^{2+} and the *E. coli* CopA transport Cu^{2+}. The two representative chimera proteins, AP (CadA/CopA) and PA (CopA/CadA), were prepared according to their transmembrane predictions (data not shown). Each of these chimera contains half of the protein (including the first four or last four transmembrane segments from either CadA or CopA proteins). In order to determine if these chimera proteins are functionally active, as well as their substrate changed or not, we have performed resistant assays. Either AP or PA chimera were transformed into proper host for these resistant assay, including for cadmium resistant assays, the AP and PA chimera were expressed in a cadmium sensitive strain, *E. coli* RW3110. When assay their copper resistance, the AP and PA chimera were transformed into *E. coli* LMGΔC194, the copper sensitive strain. The resistance assays were performed based on the standard procedures, and their results are shown below.

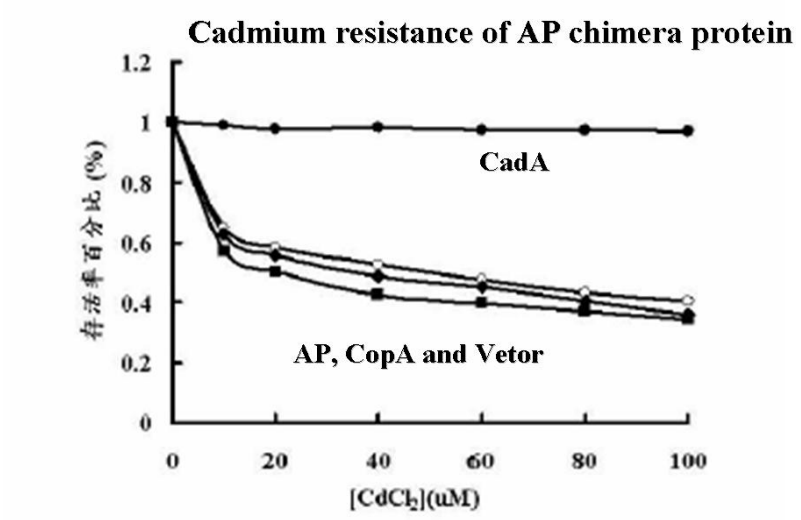

Fig 1. Cadmium resistant assays of AP chimera protein

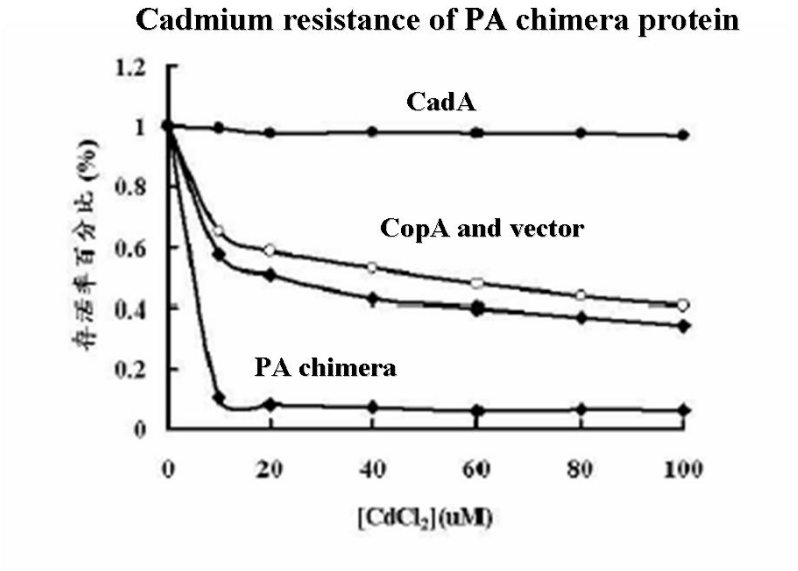

Fig 2. Cadmium resistant assays of PA chimera protein

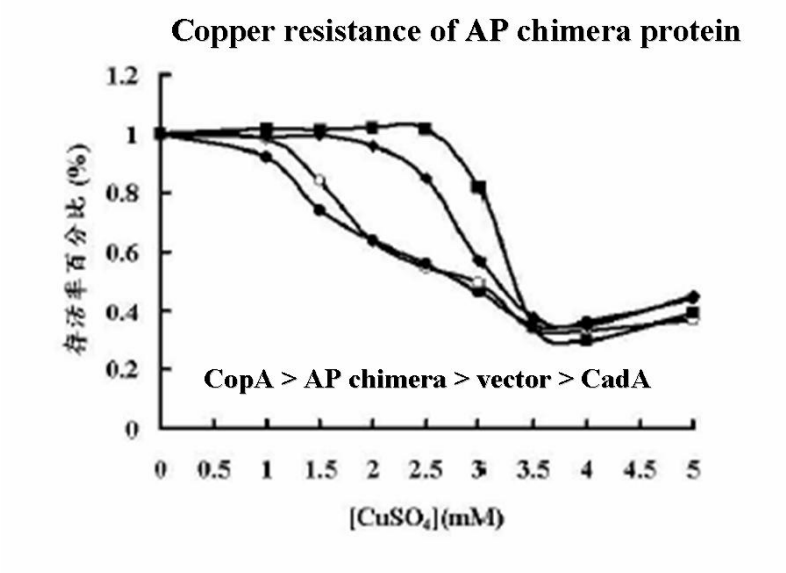

Fig 3. Copper resistant assays of AP chimera protein

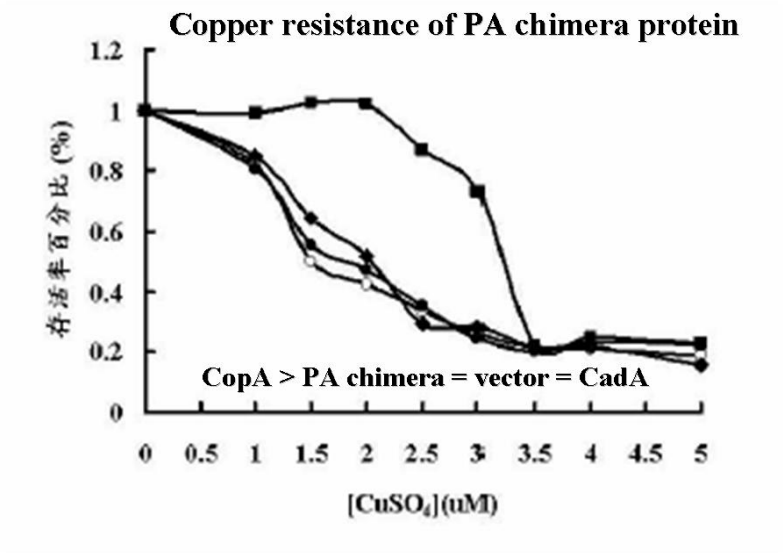

Fig 4. Copper resistant assays of PA chimera protein

Based on the results above, we found AP chimera proteins remain fairly activity to copper resistance (Fig. 3), however PA chimera protein confers no copper resistance (Fig. 4). Since CadA contains only one CXXC motif in its N-terminal, and therefore the AP chimera protein, inherited the N-terminal of CadA, contains only one CXXC motif in its N-terminus. As previous report [13], when remove the N-terminus, the mutant CopA (△N-CopA) lost its copper resistance. It is suggested that the two CXXC motifs found in CopA might play a different role and might not be important for copper recognition. Furthermore, these data also supported the previous speculation that the crucial factor for copper binding are buried within the fifth to eighth transmembrane segments [17].

On the other hand, we lost the cadmium resistance in AP chimera protein (Fig. 1), however, the PA chimera protein demonstrated a surprise hypersensitive to cadmium (Fig. 2). It is suggested that when CXXC from CadA was removed, other CXXC (from CopA in this case) can not complementary its role. As it was proposed previously [11], the N-terminal CXXC of CadA might be important as a sensor to regulate the CadA activity. In our previous findings (data not shown), when cysteine residues of N-terminal CXXC are replaced by other amino acids, we found a significant loss of transport activity was resulted, however a partial resistant activity to cadmium was existed. On the contrast, ZntA, another P_1-type ATPases, which transports zinc, cadmium and lead, when CXXC was deleted from the protein, the resistance and enzyme activity are reduced, but still functioning [12], it was then suggested CXXC is no need for ZntA's function. However, CXXC might be important for enhancing metal binding, and subsequently promote the enzyme activity [12].

In order to answer our data from PA chimera protein (Fig. 2), we proposed an alternative explanation, that is, the CXXC motif of CadA might play a role in regulating the membrane gate controlled by the membrane buried Cys-Pro-Cys tripeptide motif.

Once when the N-terminal CXXC was lost, the membrane cadmium-transduction Cys-Pro-Cys tripeptide was loosen controls, and the higher concentration of cadmium in the medium is able to diffuse through the concentration gradient and get into the AP containing cells, and a cadmium hypersensitive phenomenon was then observed (Fig 2). Whether or not the latter speculation is true, then it will require a further determination if N-terminal CXXC motif interacts with either intracellular domains in the neighborhood of the proposed membrane cadmium transduction domain, the Cys-Pro-Cys tripeptide region. And these studies are currently undertaken in this laboratory.

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四、計畫成果自評

Basically, we have done most of our proposed studies. However, due to the limited time available for this “very basic” research, some of the works, and some of the experiments are unsuccessful, and therefore it is very difficult to draw a clear-cut conclusion at this moment. A continuous effort of the subsequent years on this project will still be taken in this laboratory to complete all the proposed experiments in order to determine the role of these conserved domains found in P_1-type ATPases, and to achieve a publication for these works. In this research, we have done a significant job in preparing a series of chimera proteins. However, due to the following problems, the results from this study are not sufficient for a publication at the moment. One of the problems is we do not have the antibody to detect if all these chimera proteins are produced (however, we can still speculate the productions of these chimera from resistance changed). It is very difficult to perform the chimera cloning works, since there

are not many available restriction enzyme sites available for the recombinant DNA. Also due to the difficulty of doing either yeast two-hybrid or *E. coli* two-hybrid, we have spent a tremendous of time in working out the condition without success. The latter piece of experiments will still be working after this research period, and it should generate more data later on.