科技部補助專題研究計畫成果報告
期末報告

PriC與PriB結合性質之研究(第2年)

計 畫 類 別 ： 個別型計畫
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執 行 期 間 ： 104年08月01日至105年07月31日
執 行 單 位 ： 中山醫學大學生物醫學科學學系（所）

計 畫 主 持 人： 黃晟洋

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報 告 附 件 ： 出席國際學術會議心得報告

中 華 民 國 105 年 10 月 12 日

中 文 摘 要 ： PriC是革蘭氏陰性菌所特有的蛋白質，是複製重啟之引子合成體(replication restart primosome) 組裝與產生活性時所必需要的一員，已知若此活性遭到壓制將對細菌的生長造成極大影響。不同於PriA為首的組裝機制須靠多個相異的蛋白質協力聚集(PriA-PriB-DnaT-DnaC dependent primosome)，PriC已知可獨力組裝以PriC為首的引子合成體(PriC-dependent primosome)，使停滯的DNA複製叉重新啟動。在此研究，我們發現PriC可分別與另引子合成體蛋白質PriB以及DnaT產生交互作用力，因此這兩個不同的引子合成體組裝途徑可能是可以相互調控的。

中 文 關 鍵 詞 ： DNA修復；DNA複製；蛋白質複合體；蛋白質與DNA交互作用；蛋白質與蛋白質交互作用；抑制劑開發；PriC 蛋白質；DnaT 蛋白質

英 文 摘 要 ： PriC protein, only found in the Gram-negative bacteria, is required for assembly of the replication restart primosome. Activities of the replication restart primosome are essential for cell growth and survival. In contrast to the PriA-directed primosome using multiprotein PriA/PriB/DnaT/DnaC complex for replication restart, PriC is able to do this on its own. In this study, we identified the PriC-PriB and PriC-DnaT protein-protein interactions, suggesting a putative regulatory link between the two independent replication restart pathways.

英 文 關 鍵 詞 ： DNA Repair; DNA Replication; Protein Complexes; Protein-DNA Interaction; Protein-Protein Interactions; Inhibitor Development; PriC; DnaT.

科技部補助專題研究計畫成果報告

（□期中進度報告/■期末報告）

PriC 與 PriB 結合性質之研究

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執行機構及系所：中山醫學大學/生物醫學科學系

計畫主持人：黃晟洋
共同主持人：無
計畫參與人員：黃彥華、黃建智

本計畫除繳交成果報告外，另含下列出國報告，共 _2_ 份：
□執行國際合作與移地研究心得報告
■出席國際學術會議心得報告

期末報告處理方式：

1. 公開方式：
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2. 「本研究」是否已有嚴重損及公共利益之發現：■否 □是
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中　華　民　國　105　年 10 月 11 日

中英文摘要

PriC 是革蘭氏陰性菌所特有的蛋白質，是複製重啟之引子合成體 (replication restart primosome) 組裝與產生活性時所必需要的一員，已知若此活性遭到壓制將對細菌的生長造成極大影響。不同於 PriA 為首的組裝機制須靠多個相異的蛋白質協力聚集(PriA-PriB-DnaT-DnaC dependent primosome)，PriC 已知可獨力組裝以 PriC 為首的引子合成體(PriC-dependent primosome)，使停滯的 DNA 複製叉重新啟動。在此研究，我們發現 PriC 可分別與另引子合成體蛋白質 PriB 以及 DnaT 產生交互作用力，因此這兩個不同的引子合成體組裝途徑可能是可以相互調控的。

關鍵詞：DNA 修復；DNA 複製；蛋白質複合體；蛋白質與 DNA 交互作用；蛋白質與蛋白質交互作用；抑制劑開發；PriC 蛋白質；DnaT 蛋白質。

PriC protein, only found in the Gram-negative bacteria, is required for assembly of the replication restart primosome. Activities of the replication restart primosome are essential for cell growth and survival. In contrast to the PriA-directed primosome using multiprotein PriA/PriB/DnaT/DnaC complex for replication restart, PriC is able to do this on its own. In this study, we identified the PriC-PriB and PriC-DnaT protein-protein interactions, suggesting a putative regulatory link between the two independent replication restart pathways.

Keywords: DNA Repair; DNA Replication; Protein Complexes; Protein-DNA Interaction; Protein-Protein Interactions; Inhibitor Development; PriC; DnaT.

報告內容

(1) 研究背景 (含前言、研究目的與文獻探討)

Genetic integrity should be maintained from generation to generation for bacteria to survive [1]. DNA damage causes arrest and disassembly of the replication machinery anywhere along the DNA, leading to replication failure [2]. After DNA damage removal, DNA repair, and DNA recombination, collapsed DNA replication forks are reactivated by the replication restart primosome, a formidable enzymatic machine [3]. In Escherichia coli, the replication restart primosome is composed of PriA helicase, PriB, DnaT, PriC, DnaC, DnaB helicase, and DnaG primase. Two DnaB helicase-recruiting pathways are known to date, namely, PriA – PriB – DnaT – DnaC-dependent and PriC – DnaC-dependent systems. As shown in Fig. 1A, PriA can bind directly and assemble a primosome on the template without gaps in the leading strand, and PriC initiates the assembly of a primosome on a fork containing gaps (>5 nucleotides) in the leading strand [4].

DnaT, formerly known as protein i [5], is the third protein to be assembled in the PriA-dependent primosome [6]. Genetic analysis of E. coli DnaT suggests that it is an essential replication protein for bacterial cell growth because the dnaT822 mutant shows colony size, cell morphology, an inability to partition nucleoids properly, UV sensitivity, and basal SOS expression similar to those of priA2::kan mutants [7]. Recently, DnaT was found to possess single-stranded DNA (ssDNA)-binding activity [8]; consequently, a hand-off mechanism for PriA-dependent primosome assembly was modified [9]. DnaT consists of an N-terminal oligomerization domain with PriB-binding activity [10, 11] and a C-terminal domain with ssDNA-binding activity [8, 10, 11]. The acidic linker in DnaT is involved in the dissociation of ssDNA from the PriB – ssDNA complex [10]. Unlike PriB, which uses OB-fold to bind ssDNA [12, 13], the complex structure of DnaT reveals a novel mode to bind ssDNA using the three-helix bundle [14].

PriC is an essential initiator protein in the replication restart of the PriA-independent (PriC-directed) pathway [15]. This pathway mediated by PriC only needs minimal requirements, and its mechanism includes just three steps: (1) DNA substrate binding, (2) SSB binding and structural modulation, and (3) helicase binding [16]. PriC is divided into two domains [17]. The structure of the N-terminal domain of PriC is composed of three helices and one extended loop [18]. The basic and aromatic residues in the C-terminal domain of PriC are involved in both ssDNA and SSB binding [19].

PriB is a basic accessory protein in PriA-directed DNA replication restart primosome. It was originally discovered as an essential factor for the conversion of single-stranded circular DNA to the replicative form DNA of ϕX174 single-stranded phage in vitro. In contrast to the ϕX174 model, del(priB)302 mutant has almost wild type phenotypes, suggesting that PriB is not absolutely required for bacterial DNA replication. PriB was formerly known as the “n protein” because it can be inactivated by treatment with N-ethylmaleimide. In a PriA-PriBDnaT-dependent reaction, PriB is the second protein to be assembled in the protein-DNA complex. It stabilizes the binding of PriA to DNA hairpin and then stimulates PriA helicase activity. The PriA stimulation by PriB correlates with the ability of PriB to form a stable PriA-PriB-DNA complex. PriB also facilitates the association of DnaT with PriA. More than one PriA-PriB complex is possibly involved in the initiation of primosome formation, and the effect of PriB on the PriA-DNA

association is dependent on the DNA structure. PriB interacts with PriA, DnaT, SSB, and itself and does not interact with DnaA, DnaB, DnaC, or DnaG. The mechanisms of DnaC-DnaB complex loading by PriA-PriB-DnaT complex at the forks and then DnaB-DnaG complex formation remain unclear.

PriC is a key initiator protein in the replication restart of the PriA-independent pathway [15]. However, the mechanism of the interaction of PriC with the component of the PriA-directed primosome is still unknown. In this study, we found a direct physical interaction between DnaT, a component of PriA-directed primosome, and PriC, an initiator of PriA-independent primosome. We also found the PriC-PriB interactions. These results constitute a putative link between PriA and PriC-directed primosomes by DnaT. Further research can directly focus on determining how DnaT binds to the PriC-SSB-DNA tricomplex and regulates the PriC-dependent replication restart.

(2) 研究方法

2.1. Construction of expression plasmid of PriC

The gene encoding the putative PriC protein (KPN_00449) was amplified by PCR using genomic DNA of Klebsiella pneumoniae subsp. pneumoniae MGH 78578 as the template. The forward (5´-GGGAATTCCATATGGTGAAAACAGCTCAACTTTTA-3´) and the reverse (5´-GGGGCTCGAGGCGCGTCAGCCGCGCCAGTAC-3´) primers were designed to introduce unique NdeI and XhoI restriction sites (underlined) into PriC, permitting the insertion of the amplified gene into the pET21b vector (Novagen Inc., Madison, WI, USA) for the protein expression in E. coli BL21.

2.2. Protein expression and purification

The recombinant SSB [20], dihydropyrimidinase [21], and DnaT [8] were expressed and purified using the protocol described previously. The recombinant PriC were expressed and purified using the protocol described previously for DnaD [22]. Briefly, E. coli BL21(DE3) cells were individually transformed with the expression vector and overexpression of the expression plasmids was induced by incubating with 1 mM isopropyl thiogalactopyranoside. The cells overexpressing the protein were chilled on ice, harvested by centrifugation, resuspended in Buffer A (20 mM Tris-HCl, 5 mM imidazole, and 0.5 M NaCl, pH 7.9) and disrupted by sonication with ice cooling. The protein purified from the soluble supernatant by Ni2+-affinity chromatography (HiTrap HP; GE Healthcare Bio-Sciences, Piscataway, NJ, USA) was eluted with Buffer B (20 mM Tris-HCl, 250 mM imidazole, and 0.5 M NaCl, pH 7.9) and dialyzed against a dialysis buffer (10 mM Na2HPO4, 2mM KH2PO4, 137 mM NaCl, 2.7 mM KCl, and 10% glycerol, pH 7.4; Buffer C). Purity of these proteins remained greater than 97 % as determined by Coomassie-stained SDS-PAGE (Mini-PROTEAN Tetra System; Bio-Rad, CA, USA).

2.3. Co-purification of DnaT–PriC complex using the heparin column

Co-purification of DnaT-PriC complex using the heparin HP column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) was carried out by the AKTA-FPLC system (GE Healthcare Bio-Sciences, Piscataway,

NJ, USA). Briefly, the purified DnaT protein solution (3.1 mg/mL) was mixed with the PriC protein solution (4 mg/mL). After dialysis against Buffer D (20 mM potassium phosphate and 50 mM NaCl, pH 7.0), the protein solution containing PriC and DnaT was directly applied to the heparin HP column and eluted with a linear NaCl gradient from 0.05 to 1.0 M with Buffer D. Total elution volume was 300 mL. The peak fractions from this chromatographic step were collected and analyzed by Coomassie-stained SDS-PAGE.

2.4. Fluorescence-quenching measurement

Fluorescence titrations were performed in a spectrofluorimeter (Hitachi F-2700; Hitachi High-Technologies, Tokyo, Japan) as described previously [21-24]. The excitation and emission of tryptophan fluorescence were detected at 297 and 340 nm, respectively. The PriC protein solution (1 μM) in 2 mL Buffer C was titrated with increasing quantities of dT20 oligonucleotide. After the addition of ssDNA, the complex solution was equilibrated for 300 s until no fluorescence change could be observed. Tryptophan fluorescence quenching was used to measure the diminution of fluorescence between the ssDNA-free and the ssDNA-bound proteins.

2.5. Gold nanoparticle assay

A protein–protein interaction within PriC–DnaT was rapidly analyzed based on the intrinsic optical properties of the gold nanoparticles [22, 25]. Other proteins such as SSB (1 μM) and dihydropyrimidinase (1 μM) were also used in this assay. The commercialized kit of Ni2+-NTA SAM nanoparticles (SmarticleParticles) was purchased from Minerva Biotechnologies (Waltham, MA, USA). The 75-μL coupling sample 1 contains 50 μL of PriC (1 μM) and 25 μL of Ni2+-NTA SAM nanoparticles, whereas the 75-μL coupling sample 2 contains 50 μL of DnaT (1 μM) and 25 μL of Ni2+-NTA SAM nanoparticles. Sample 1 was mixed with sample 2 for 1–10 min. Red/pink indicates a homogeneous suspension, and purple indicates that particles are drawn close together through protein–protein interactions.

2.6. Surface plasmon resonance analysis (SPR)

SPR was conducted using the protocol described previously for DnaC helicase [26] and DnaD protein [22]. PriC was individually immobilized on Series S sensor chips CM5 (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The DnaT-binding experiments were carried out at 293K using a Biacore T200 (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) with running buffer (40 mM Tris, 200 mM NaCl, and 0.05% Tween-20 at pH 8.0). DnaT solutions were diluted in the running buffer to final concentrations of 500, 250, 125, 62, and 31 nM. Diluted samples were injected in duplicate over each immobilized protein for 120 s at a flow rate of 30 μL/min. The running buffer was then flushed for 300 s at a flow rate of 30 μL/min. Finally, the chip surface was regenerated by injecting 2 M MgCl2 buffer for 60 s at a flow rate of 30 μL/min. Control samples were used to monitor the sensor chip surface stability, demonstrating reproducibility throughout the duration of the experiments. The estimated Kd values were derived by fitting the association and dissociation signals with a 1:1 (Langmuir) model using the Biacore T200 Evaluation Software.

2.7. Pull-down assay

PriC Protein was incubated at 25 ℃ for 20 min with PriB protein in Buffer C. Nickel-NTA agarose beads (GE Healthsciences) were added to each sample, incubated for 2 min, and centrifuged for 1 min at 6000g. The beads were washed several times with Buffer C. Proteins were eluted with Buffer B and visualized by Coomassie stained SDS–PAGE.

(3) 結果與討論

3.1. Purification of PriC

The gene KPN_00449 encoding for the putative PriC was PCR-amplified using the genomic DNA of *K. pneumoniae* as template. This amplified gene was then ligated into the pET21b vector for protein expression. PriC was heterologously overexpressed in *E. coli* and then purified from the soluble supernatant by Ni2+-affinity chromatography. Pure PriC protein was obtained in this single chromatographic step with an elution of Buffer B and then dialyzed against a dialysis buffer (Buffer C). The purified recombinant *K. pneumoniae* PriC protein in Buffer B precipitated in three days, probably because of its aggregated features, which are also found in *E. coli* PriC protein [17]. The dialysis of *K. pneumoniae* PriC solution against Buffer C was found useful in improving the stability and solubility of PriC. After dialysis, precipitation of *K. pneumoniae* PriC was observed in more than two weeks. Approximately 2 mg of purified PriC protein was obtained from 1 L of *E. coli* cell culture. Purified DnaT protein was obtained using the protocol described previously [8, 11].

3.2. Sequence analysis of PriC

Based on the known nucleotide sequence, the predicted PriC monomer protein has a length of 176 amino acid residues and a molecular mass of ~21 kDa. Fig. 1B shows the alignment consensus of 140 sequenced PriC homologs by ConSurf [23, 27, 28], revealing the degree of variability at each position along the primary sequence. Highly variable amino acid residues are colored teal, whereas highly conserved amino acid residues are burgundy. A consensus sequence was established by determining the most commonly found amino acid residue at each position relative to the primary sequence of *K. pneumoniae* PriC. The residues Arg107, Lys111, Phe118, Arg121, and Lys165 involved in ssDNA binding in E. coli PriC [19] are Arg108, Arg112, Phe119, Arg122, and Lys166 in *K. pneumoniae* PriC, respectively. Except for Lys166, these residues are conserved in most PriC families (Fig. 1B).

3.3. Interaction of *K. pneumoniae* PriC and ssDNA

We examined the quenching fluorescence intensity of tryptophan in *K. pneumoniae* PriC upon addition of ssDNA. *K. pneumoniae* PriC has four tryptophan residues (Trp75, Trp94, Trp99, and Trp219), allowing ssDNA binding analysis through tryptophan fluorescence quenching. The protein displayed strong intrinsic fluorescence with a peak wavelength of 340 nm when excited at 297 nm, which is consistent with tryptophan fluorescence. Upon the addition of ssDNA, fluorescence quenching of *K. pneumoniae* PriC was

observed. The intrinsic fluorescence was progressively quenched as dT20 was titrated into the *K. pneumoniae* PriC solution (Fig. 2A). Upon adding a saturating quantity of ssDNA, the intrinsic fluorescence at 340 nm was quenched by 50%. Using these fluorescence data, we calculated the binding stoichiometry between K. pneumoniae PriC and ssDNA. The saturation curves suggest that the binding is stoichiometric at 0.44 *K. pneumoniae* PriC monomer per 20-mer ssDNA if enough binding sites were present in the DNA for all *K. pneumoniae* PriC molecules to bind. Therefore, the binding-site size of *K. pneumoniae* PriC monomer estimated from fluorescence quenching is approximately 9 nt (20 × 0.44 = 8.8). The binding-site size of *E. coli* PriC has been reported to be 7-9 nt [17]. Thus, PriC from *K. pneumoniae* and *E. coli* have similar binding-site size to ssDNA.

3.4. Interaction of PriC with DnaT

Although the role of DnaT in the recruitment of DnaB helicase has been proposed, nothing is known on the interaction of DnaT with PriC. We carried out gold nanoparticle assay to rapidly screen the interaction mechanism of PriC with DnaT. SSB, known to interact with PriC [16, 19], was also included in this assay as a positive control. As histidine-tagged proteins, SSB, PriC, and DnaT can be immobilized on commercialized Ni^{2+}-NTA SAM nanoparticles. Protein-loaded nanoparticles retain the same red color as the unmodified SAM-coated nanoparticles [25]. However, the solution turns purple when gold nanoparticles aggregate, indicating the protein–protein interactions [22]. The gold nanoparticle assay showed that the solutions of PriC–DnaT and PriC–SSB changed from red to purple (Fig. 2B), whereas that of PriC–dihydropyrimidinase (dht) [21], as a negative control, maintained its red color. Thus, PriC was identified as a DnaT-interacting protein for the first time.

3.5. Co-purification of PriC–DnaT complex using the heparin column

The heparin column is generally useful for purifying proteins with an affinity for heparin, such as ssDNA-binding proteins like SSB [29]. Both DnaT and PriC can bind the heparin column, as shown in Fig. 2C. However, their elution volumes are different. DnaT can be eluted with 710 mM NaCl with an elution volume of 208 mL, whereas PriC can be eluted with 690 mM NaCl with an elution volume of 202 mL (a linear NaCl gradient from 0.05 M to 1.0 M with a total elution volume of 300 mL). We carried out co-purification of PriC and DnaT using the heparin column to establish further whether PriC can form a complex with DnaT. Purified DnaT (3.1 mg/mL) and PriC (4 mg/mL) were mixed to form a complex by incubation at room temperature for 15 m. The protein solution containing PriC and DnaT was then directly applied to the heparin HP column, and the new peak fractions from this chromatographic step were collected and verified by Coomassie-stained SDS-PAGE (Fig. 2C). The PriC–DnaT complex eluted with 440 mM NaCl (with an elution volume of 124 mL), significantly earlier than either DnaT or PriC. Thus, the PriC–DnaT complex was formed and could be co-purified.

3.6. SPR

In this study, we identified the interaction between PriC and DnaT using the gold nanoparticle assay and

the heparin column. SPR was used to provide experimental evidence for estimating the binding affinity between these proteins and thus quantitatively characterize PriC–DnaT interaction further. PriC was immobilized on a sensor chip (as a ligand), and the DnaT solution (as an analyte) was passed over the sensor chip in a microfluidic chamber. Fig. 2D shows the SPR results at various DnaT concentrations. The Kd value of PriC bound by DnaT, calculated from the equilibrium binding isotherms using a simple binding model (a 1:1 Langmuir binding model), was $2.9 \pm 0.3 \times 10^{-8}$ M.

3.7. Pull-down assay for PriC-PriB interaction

To verify direct interaction of PriC with PriB, we performed pull-down experiments. If there is physical interaction between PriCT and PriB, untagged PriB(dHis) will be eluted with His-tagged PriC in this assay. We found the co-elution, and identified their interactions. We will find more evidences for this identification.

3.8. Possible role of DnaT in PriC-directed primosome assembly

We recently identified that DnaT is a kind of ssDNA binding protein [8] and then modified the hand-off mechanism for PriA-directed primosome assembly [9]. In the current study, we found that DnaT can also bind PriC (Fig. 2). PriC is one of the two essential initiators (another one is PriA) in DnaB helicase-recruiting pathways for DNA replication restart at the stalled replication fork containing large gaps. Given that many bacteria have no recognizable homologue of DnaT [9], and because PriC can catalyze DnaB loading alone [30], we proposed that DnaT serves as an accessory protein for regulating the translocase/helicase activity or loading efficiency of replicative DnaB helicase in both PriA- and PriC-directed primosome assemblies. However, this speculation must be confirmed by additional biophysical and biochemical studies.

The PriC-directed primosome assembly has been proposed [16], wherein (i) PriC recognizes and binds to a stalled replication fork through interactions with ssDNA and SSB, and (ii) the DnaB/C complex is loaded after specific structural conformational change in the SSB–DNA complex. On the basis of our results that DnaT is a ssDNA-binding and PriC-binding protein, the PriC-directed primosome assembly is updated (Fig. 3), such that (i) PriC recognizes and binds to the replication fork through interactions with ssDNA and SSB, and (ii) DnaT may join a PriC–DNA complex or a PriC–SSB–DNA ternary complex to regulate the DnaB/C complex loading. This modified model explains the binding abilities of DnaT to ssDNA and the initiator PriC, being a loader protein for DnaB helicase recruiting. Whether or not its partner protein, PriB, also binds to PriC-directed primosome via the link with DnaT is still unclear. A large number of complex structures of DnaT will enhance our understanding of the mechanisms for PriA- and PriC-directed primosome assemblies. Given that SSB and DnaT are both ssDNA- and PriC-binding proteins, further research can directly focus on determining whether DnaT is a competitor or an enhancer for SSB binding to PriC.

3.9. Publication

Part of results has been published on ***Biochem. Biophys. Res. Commun.***, 477, 988-992, 2016.

Figure 1

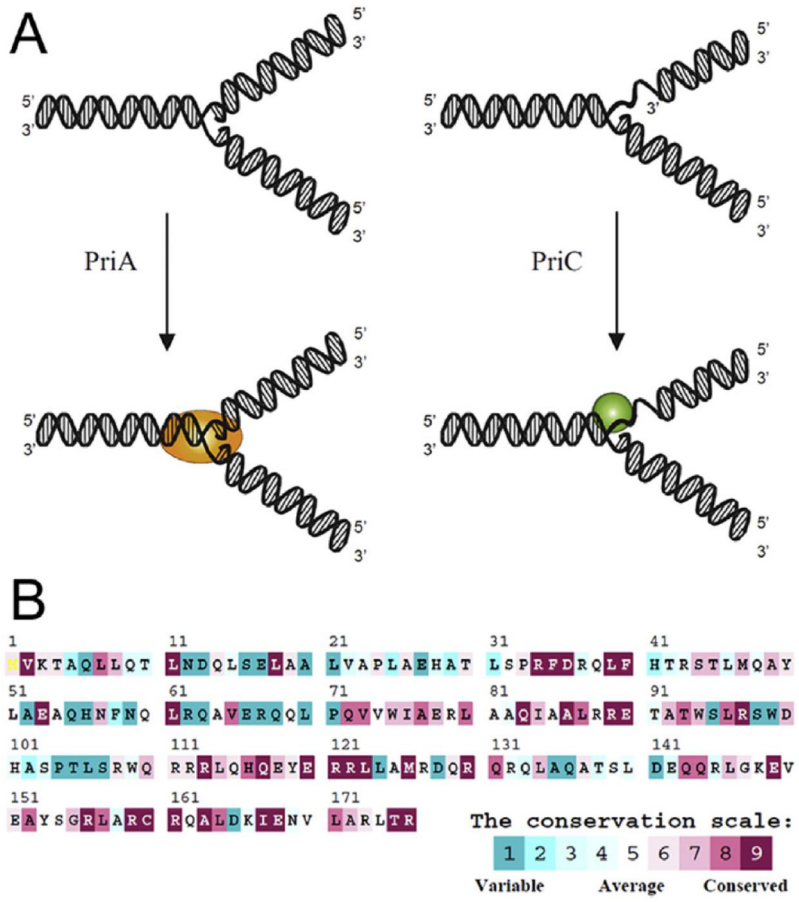

Figure 1. (A) Two DnaB helicase-recruiting pathways for DNA replication restart at the stalled replication fork in vitro. The PriA-directed pathway (i.e., PriA-PriB-DnaT-DnaC-dependent reaction) preferentially uses fork structures without gaps in the leading strand, whereas the PriC-directed pathway (i.e., PriC-DnaC-dependent system) preferentially uses fork structures containing large gaps (>5 nucleotides) in the leading strand. (B) An alignment consensus of 160 sequenced PriC homologs by ConSurf. It reveals the degree of variability at each position along the primary sequence. Highly variable amino acid residues are colored teal, whereas highly conserved amino acid residues are burgundy. A consensus sequence was established by determining the most commonly found amino acid residue at each position relative to the primary sequence of K. pneumoniae PriC. The residues Arg107, Lys111, Phe118, Arg121, and Lys165 involved in ssDNA binding in E. coli PriC are Arg108, Arg112, Phe119, Arg122, and Lys166 in K. pneumoniae PriC, respectively. Except for Lys166, these residues are conserved in most PriC families

Figure 2

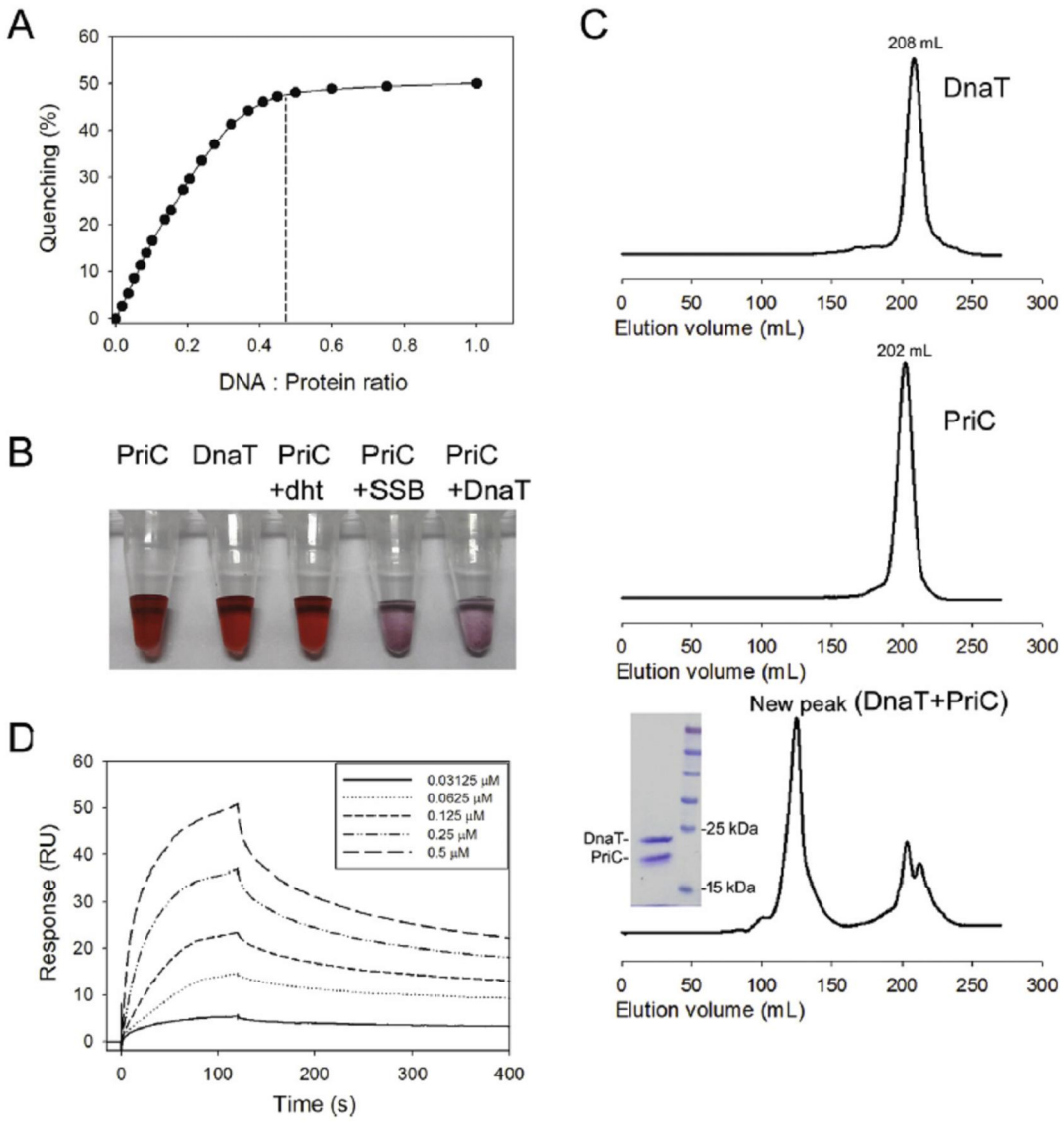

Figure 2. (A) Fluorescence quenching. The excitation and emission of tryptophan fluorescence were detected at 297 and 340 nm, respectively. The PriC protein solution (1 μM) in 2 mL Buffer C was titrated with increasing quantities of dT20 oligonucleotide. After the addition of ssDNA, the complex solution was equilibrated for 300 s until no fluorescence change could be observed. (B) The gold nanoparticle assay. PriC protein solution, DnaT protein solution, mixture of PriC and dht (dihydropyrimidinase), mixture of PriC and SSB, and mixture of PriC and DnaT were analyzed. The gold nanoparticle assay showed that the solutions of PriC–DnaT and PriC–SSB changed from red to purple, indicating the protein–protein interactions. (C) Co-purification of PriC–DnaT complex using the heparin column. Both DnaT (with an elution volume of 208 mL) and PriC (with an elution volume of 202 mL) can bind the heparin column. We carried out co-purification of PriC and DnaT using the heparin column to establish further whether PriC can form a complex with DnaT. Purified DnaT (3.1 mg/mL) and PriC (4 mg/mL) were mixed to form a complex, and the protein solution was then directly applied to the heparin HP column. The new peak fractions (with an elution volume of 124 mL) from this chromatographic step were collected and verified by Coomassie-stained SDS-PAGE. Thus, the PriC–DnaT complex was formed and could be co-purified by the heparin HP column. (D) SPR analysis of the DnaT–PriC interaction. The estimated Kd value was derived by fitting the association and dissociation signals with a 1:1 (Langmuir) model using the Biacore T200 Evaluation Software.

Figure 3

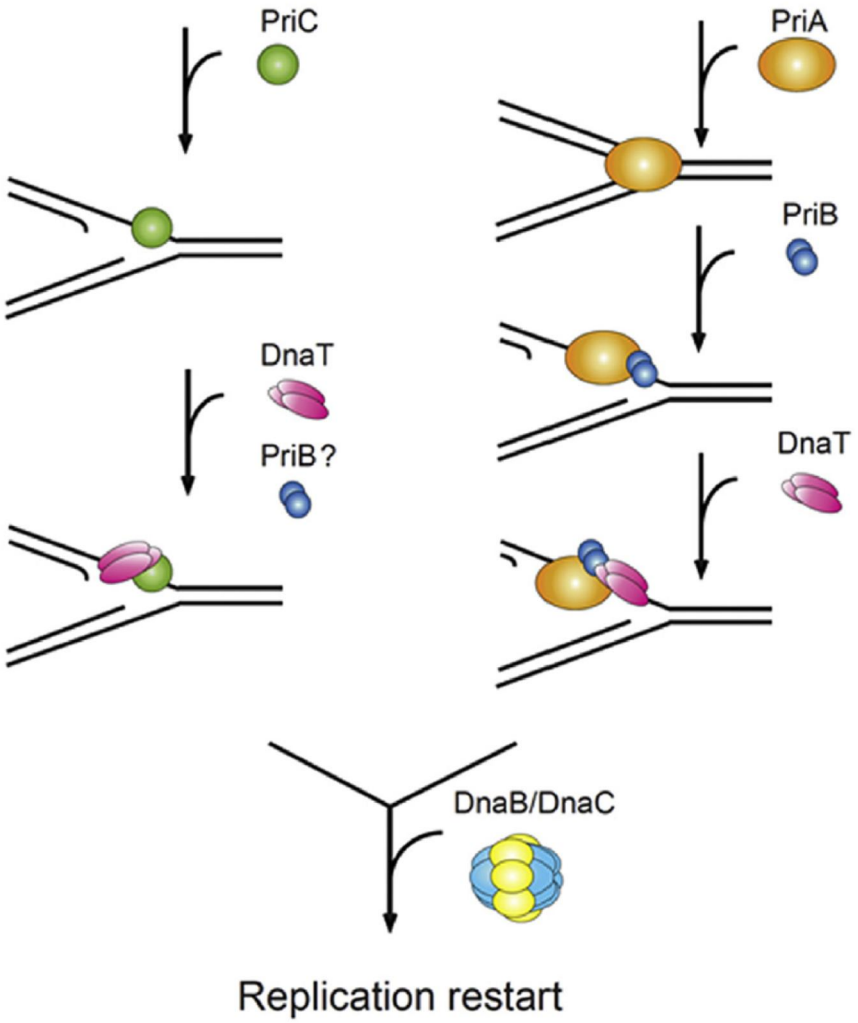

Figure 3. A putative link between PriA and PriC-directed primosomes by DnaT. Two DnaB helicase-recruiting pathways are known: PriA–PriB–DnaT–DnaC-dependent and PriC–DnaC-dependent systems [8, 9, 16]. On the basis of our results that DnaT is a ssDNA-binding and PriC-binding protein, the PriC-directed primosome assembly is updated, such that (i) PriC recognizes and binds to the replication fork through interactions with ssDNA and SSB, and (ii) DnaT may join a PriC–DNA complex or a PriC–SSB–DNA ternary complex to regulate the DnaB/C complex loading. For clarity, SSB is not shown in both pathways in this figure. This modified model explains the binding abilities of DnaT to ssDNA and the initiator PriC, being a loader protein for DnaB helicase recruiting. Whether or not its partner protein, PriB, also binds to PriC-directed primosome via the link with DnaT is still unclear.

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科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否有嚴重損及公共利益之發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■ 達成目標

說明：我們已證明相關的交互作用，並已發表 5 篇 SCI 論文。

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

目前共發表 5 篇，本人皆為通訊作者，剩下仍待整理與撰寫中。

(1) Huang, C.C., **Huang, C.Y.*** (2016) DnaT is a PriC-binding protein. ***Biochem. Biophys. Res. Commun.***, 477, 988-992. (SCI)

(2) Huang, Y.H., Lien, Y., Huang, C.C., **Huang, C.Y.*** (2016) Characterization of *Staphylococcus aureus* primosomal DnaD protein: Highly conserved C-terminal region is crucial for ssDNA and PriA helicase binding but not for DnaA protein-binding and self-tetramerization. ***PLoS One***, 11, e0157593. (SCI)

(3) **Huang, C.Y.*** (2015) Inhibition of a putative dihydropyrimidinase from *Pseudomonas aeruginosa* PAO1 by flavonoids and substrates of cyclic amidohydrolases. ***PLoS One***, 10, e0127634. (SCI)

(4) Huang, Y.H., **Huang, C.Y.*** (2015) Creation of a putative third metal binding site in type II dihydroorotases significantly enhances enzyme activity. ***Protein Pept. Lett***., 22, 1117-1122. (SCI)

(5) Huang, Y.H., Huang, C.C., Chen, C.C., Yang, K.J., **Huang, C.Y.*** (2015) Inhibition of *Staphylococcus aureus* PriA helicase by flavonol kaempferol. ***Protein J***., 34, 169-172. (SCI)

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值，如已有嚴重損及公共利益之發現，請簡述可能損及之相關程度。

研究成果已發表數篇論文，其他結果仍持續整理並撰寫中，希望能對新型抗生素的研發做出貢獻。

科技部補助專題研究計畫出席國際學術會議心得報告

日期：105 年 10 月 11 日

計畫編號	MOST 103-2320-B-040-018-MY2		
計畫名稱	PriC 與 PriB 結合性質之研究		
出國人員 姓名	黃建智	服務機構 及職稱	中山醫學大學/生物醫學科學系/ 研究生(計畫兼任助理)
會議時間	105 年 6 月 7 日至 105 年 6 月 9 日	會議地點	日本/福岡
會議名稱	(中文)第 16 回日本蛋白質科學年會 (英文) The 16th Annual Meeting of the Protein Science Society of Japan		
發表題目	(中文)DnaD 的 C 端區域對單股 DNA 與 PriA 解旋酶的結合是重要的 (英文) The C-terminal region of DnaD protein is crucial for ssDNA and PriA helicase binding		

一、參加會議經過

The 16th Annual Meeting of the Protein Science Society of Japan was held from Jun 7 to 9, 2016 in Fukuoka, Japan. As the meeting is mainly based on biophysics for the main shaft, so lots of the participating researchers are in the fields of macromolecular interactions in which I am rather interested. In the lecture sections of Prof. Kenji Iwasaki, Prof. Takashi Fujii, and Prof. Ryo Nitta, I am deeply attracted to learn more about new and efficient strategies to develop my research scenario. I also contributed a post presentation (1P-032) at Day 1 (June 7). The title is “The C-terminal region of DnaD protein is crucial for ssDNA and PriA helicase binding.” Part of results for presentation in this conference has been published on PLoS One, 11, e0157593, 2016.

二、與會心得

I would briefly elucidate those summaries of my most impressive lectures as following:

1). The talk by Dr. Luo Fangjia, entitled “Processing of XFEL still images with a reference oscillation data set for crystal structural analyses of cytochrome c oxidase”, provides a method to analyze the structure. In this method, serial femtosecond rotation crystallography (SF-ROX), a crystal mounted on a

goniometer-head is exposed by the X-ray pulse and translates for the next exposure to obtain serial diffraction images with an equal rotation interval. To obtain high quality data set from SF-ROX diffraction images, data processing procedure was developed. A reference intensity data set is obtained by the oscillation method using crystals grown in the same batch as those used in SF-ROX method. Fix cell constant to those of SF-ROX data before processing. Crystal orientation parameters are refined to reduce the difference between I of the reference data and I/P of the target crystal, where P stands for partiality reflection. P is a function of the distance of a reciprocal lattice point from the reflection sphere. Once orientation parameters converge well in the refinement procedure, P is estimated and the observed intensity is evaluated by the formula,I/P.

2). The talk by Dr. Mariko Nagata, entitled “How do the RecJ/Cdc45 proteins work for the genome stability in Archaea?”, in which they studied the genome stability of Archaea. RecJ/Cdc45-like sequence is conserved in the three domains of life. The bacterial RecJ protein plays important roles in a number of DNA repair and recombination pathways, whereas the cell division cycle 45 protein (Cdc45) is a member of the Cdc45-MCM-GINS (CMG) complex, working as a replicative helicase. The two RecJ-like proteins, RecJ1 and RecJ2, from *Thermococcus kodakarensis*, were characterized. Two RecJs showed different nuclease activity in vitro. RecJ1 formed complex with MCM and GINS, suggesting the possibility of the CMG-like formation. However, any nuclease activity of Cdc45 has not been reported to date, and therefore, the distance nuclease activity of RecJ1 suggests the different mechanisms for the replication fork progression between Archaea and Eukarya.

3). The talk by Dr. Eriko Aoki, entitled “Transmembrane domain of an adhesion is translocated into outer membrance without its signal sequence”. The envelope of Gram-negative bacteria is composed of two distinct membranes, the inner membrane (IM) and the outer membrane (OM). The IM enclose the cytoplasm and is surrounded by the OM. These membranes are separated by the periplasmic space. Proteins embedded in OM are essential for passage of nutrients and wastes or virulence. These outer membrane proteins (OMPs) are synthesized in cytoplasm as precursor proteins with an N-terminal signal sequence. The signal sequence is believed to be required for the translocation across IM via Sec system. The signal sequence-less OMP is deficient in the ability to assemble into OM, and several OMPs expressed without signal sequence have been known to accumulate into inclusion bodies in cytoplasm. The transmembrane β-barrel domain of Haemophilus Influenzae adhesion (Hia) in *E.coli* was expressed and purified in high yield. It do not form inclusion bodies enough to be observed on SDS-PAGE with Coomassies Brilliant Blue staining. The sucrose gradient centrifugation experiment indicated that the transmembrane domain was found in OM as an assembled form.

4). The talk by Dr. Thomas P Halsted, entitled “The damage-free crystallographic study of denitrifying enzymes”. During regular synchrotron radiation crystallography (SRX), protein metal centres undergo photoreduction and their ligand geometry is perturbed. Copper nitrite reductase (CuNiR) contains two copper centres, one T1Cu and one T2Cu, and catalyses the one electron reduction of nitrite to nitrous oxide. The T1Cu site acts to accept electron from either blue copper protein or c-type cytochromes. Complete electron transfer occurs from the T1Cu site only occurs when nitrite is bound at the T2Cu, suggesting a proton-coupled electron transfer (PCET) mechanism. This ordered mechanism is disputed however by kinetic studies, displaying evidence for a random mechanism. To fully elucidate the mechanism of CuNiR and investigate the nature of electron transport (ET) complex, high resolution native structures are required that collection at synchrotron sources. The collection of native CuNiR datasets, growing CuNiR-electron donor co-crystals and futures XFEL experiments to provide new insights into mechanism of CuNiR were discussed.

5). The talk by Dr. Ryuichiro Terada, entitled “Hybrid initio quantum mechanism analysis of O2-binding to the CuB-Fea3 binuclear center of cytochrome c oxidase”. Cytochrome c oxidase, which is

the terminal enzyme of the electron transport chain, catalyzes an oxygen molecule to two water molecules, thus generating the driving force for proton pumps. This catalytic reaction (initiated from the reduce state) starts from binding of ligand (oxygen molecule) to the catalytic center of CcO, i.e., the binuclear center (BNC), which is composed of the CuB and heme a3 sites. To investigate the ligand recognition, the hybrid ab initio Quantum Mechanics (QM)/Molecular Mechanics (MM) Molecular Dynamics (MD) simulations was performed, which is one of the most advance computational molecular science techniques. As a result of the analysis, the binding of either O_2 or CO ligand to Fe of heme a3 induced the dynamical translocations of heme a3 was found, where the displacements were evolved within the heme plane. In the X-ray structures of CcOs in the complex with and without the ligands (CO and NO), the two-state static displacement of heme a3 were previously reported. The result from hybrid ab initio QM/MM MD simulations indicate that the anion acid residues in the vicinity of the BNC also modulate the configurations of the ligands, thus contributing to the catalytic reaction.

三、發表論文全文或摘要

ID: 1P-032

The C-terminal region of DnaD protein is crucial for ssDNA and PriA helicase binding

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DnaD is a replication restart primosomal protein that reinitiates DNA replication in bacteria. In this study, we characterized the DNA-binding properties of DnaD from Staphylococcus aureus (SaDnaD) by using electrophoretic mobility shift analysis with a series of single-stranded DNA (ssDNA) homopolymers, fluorescence quenching, fluorescence resonance energy transfer assay, bioinformatic modeling tools, and three deletion mutant proteins, namely, SaDnaD1-195, SaDnaD1-200, and SaDnaD1-204. Protein–protein interaction within SaDnaD–SaPriA was also analyzed using surface plasmon resonance (SPR) and gold nanoparticle assays. SaDnaD forms distinct complexes with ssDNA of different lengths and stoichiometries (size of binding site) of 29 ± 2 nucleotides (nt) per tetramer. In fluorescence titrations, SaDnaD bound to ssDNA with a binding-site size of approximately 27 nt. The stable complex of SaDnaD1-195, SaDnaD1-200, and SaDnaD1-204 with ssDNA dT40 was not detectable, indicating that the C-terminal region of SaDnaD (especially aa 205–228) is significantly crucial for ssDNA binding. Results from SPR and gold nanoparticle assays revealed that SaDnaD1-195 cannot interact with SaPriA. In addition, SaDnaD1-195 did not stimulate the ATPase activity of SaPriA. Basing on these results, we conclude that the highly conserved C-terminal region of DnaD is crucial for both ssDNA and PriA helicase binding.

四、攜回資料名稱及內容

The 16th Annual Meeting of the Protein Science Society of Japan abstracts book.

科技部補助專題研究計畫出席國際學術會議心得報告

日期：104 年 10 月 12 日

計畫編號	MOST 103-2320-B-040-018-MY2		
計畫名稱	PriC 與 PriB 結合性質之研究		
出國人員 姓名	黃晟洋	服務機構 及職稱	中山醫學大學/生物醫學科學系/ 副教授
會議時間	104 年 6 月 30 日至 104 年 7 月 02 日	會議地點	日本/東京
會議名稱	(中文)第 67 回日本細胞生物學會大會 (英文) The 67th Annual Meeting of the Japan Society for Cell Biology		
發表題目	(中文)類黃酮與環化胺水解酶受質對二氫嘧碇水解酶的抑制 (英文) Inhibition of dihydropyrimidinase by flavonoids and substrates of cyclic amidohydrolases		

一、參加會議經過

The 67th Annual Meeting of the Japan Society for Cell Biology was held from Jun 30 to Jul 02, 2015 in Tokyo, Japan. In the lecture sections of Prof. Randy Schekman, and Prof. Yoshihiro Yoneda, I am deeply attracted to learn more about new and efficient strategies to develop my research scenario. I also contributed a post presentation (1P-028). The title is “Inhibition of dihydropyrimidinase by flavonoids and substrates of cyclic amidohydrolases” (it has been published on *PLoS One*, 10, e0127634, 2015)

二、與會心得

I would briefly elucidate those summaries of my most impressive lectures as following:

1). The talk by Dr. Masato Koike, entitled “Cellular localization and tissue distribution of endogenous DECP1 protein”, discusses the latest topics as to where and how the DECP1 is located. Upon induction of autophagy the isolation membrane forms a phagophore. Recently, LC3-positive isolation membranes were found to emerge from a DFCP1-positive, ER-associated compartment called the omegasome. Analyses of the precise behavior and cellular distribution of DECP1 normal and starved conditions are important for a better understanding of phagophore and/or autophagosome formation. The antibody recognized both human and

mouse DFCP1 protein. In HeLa cells under normal conditions, immunoreactivity for DECP1 was found dotted or tubular along Tomo20-positive filamentous mitochondria and was only partially co-localized in the ER or Golgi apparatus. In cells expressing mCherry/GFP-DFCP1, the distribution pattern of endogenous DFCP1 was altered and adjusted to that of mCherry/GFP-DFCP1, indicating that the distribution of DFCP1 may be strictly regulated by the express amount of DFCP1 protein within a cell. Moreover, under starved conditions, distinct DFCP1-positive structure became more dotted and scattered in the cytoplasm, while part of the LC3-positive antophagosomes were immunopositive for DFCP1. Western blotting using various mouse tissues revealed that DFCP1 was ubiquitously express but in a tissue-dependent manner, and was high in CNS tissue. Immunopositive for endogenous DFCP1 in the primary cortical neurons at 10 DIV was partially co-localized with that for the ER, Golgi apparatus, and mitochondria in the somatodendritic portion, and with that for mitochondria in the pre-synaptic region. There results indicate that an antibody raised against DFCP1 could be a useful tool in explaining the mechanism of phagophore formation from omegasome compartments.

2). The talk by Dr. Masahito Tanaka, entitled “Examination of dynamics of cell membrane in migrating cells”, in which they developed a technique to examine the dynamics of cell membrane in migrating cells. Most of eukaryotic motile cells migrate by ameboid movement. Dynamics of cell membrane is considered to play an important role in cell migration. The dynamics of cell membrane is closely related to the cell migration mechanism. After the cell membrane was fluorescently stained, photobleaching was performed at paets of both dorsal ventral membrane in migrating cells. Both bleached regions were stationary relatively to the substratum, moving rearward as the cell migrated, indicating that “tank” model is more preferable. “Tank” model predicts that the addition of membrane by exocytosis at the leading edge and the uptake of the membrane by endocytosis at the rear of migrating cell.

3). The talk by Dr. Kohji Yamada, entitled “Cell surface expression of importin α1 in cancer and its functional significance”, in which they established importin α1 expression and its functional significance. Importin α1, known as karyopherin (KPNA) 2, has a central role in nucleocytoplasmic transport. Importin α1 is overexpressed in several types of cancers, including hepatocellular carcinoma. In immunofluorescence study, importin α1 was found to be expressed at the cell surface as well as the cytoplasm and the nucleus in cancer cell lines (HepG2 and HCT116). Flowcytometric analysis also showed that many typed of cancer cell lines were positive for the cell surface importin α1, whereas importin α1 at the cell surface was not detected in normal cells. In living cell surface importin α1-positive cells, recombinant importin α1 and NLS-fused proteins were associated with the cell surface, suggesting that importin α1 at the cell surface was functional. Furthermore, treatment with recombinant importin α1 increased amount of importin α1 was blocked using specific monoclonal antibody, proliferation of HepG2 and HCT116 cells was significantly inhibited. These data show that cell surface expression of importin α1 may have an important role in cancer.

4). The talk by Dr. Yueh-Chien Lin, entitled “The function of the small GTPase Arf6 in lymphangiogensis”, in which they established the function of the small GTPase Arf6. The mammalian small GTPase ADP-ribosylation factor (Arf) family consists of six related gene products, Arf1-6, which are divided into three classes based on the sequence homology. Class I include Arf1, Arf2 and Arf3, class II Arf4 and Arf5, and class III Arf6. The sole member of class III, Arf6, localizes to the plasma membrane and endosomal compartments, and plays pivotal roles in a wide variety of cellular events, including endocytosis, exocytosis, actin cytoskeleton reorganization. Arf6 knockout(Arf6-/-) mouse embryos exhibited severe skin edema at the midgestation stage and impairment in the formation of lymphatic vessel network. Knockdown of Arf6 in primary cultured lymphatic endothelial cells (LECs) inhibited the in vitro capillary tube formation and cell migration induced by VEGF-C. There results suggest that Arf6 is essential for the dynamic behavior of LECs, thereby playing a crucial role in developmental lymphangiogenesis.

三、發表論文全文或摘要

ID: 1P-028

Inhibition of dihydropyrimidinase by flavonoids and substrates of cyclic amidohydrolases

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Dihydropyrimidinase is a member of the cyclic amidohydrolase family, which also includes allantoinase, dihydroorotase, hydantoinase, and imidase. These metalloenzymes possess very similar active sites and may use a similar mechanism for catalysis. However, whether the substrates and inhibitors of other cyclic amidohydrolases can inhibit dihydropyrimidinase remains unclear. This study investigated the inhibition of dihydropyrimidinase by flavonoids and substrates of other cyclic amidohydrolases. Allantoin, dihydroorotate, 5-hydantoin acetic acid, acetohydroxamate, orotic acid, and 3-amino-1,2,4-triazole could slightly inhibit dihydropyrimidinase, and the IC_{50} values of these compounds were within the millimolar range. The inhibition of dihydropyrimidinase by flavonoids, such as myricetin, quercetin, kaempferol, galangin, dihydromyricetin, and myricitrin, was also investigated. Some of these compounds are known as inhibitors of allantoinase and dihydroorotase. Although the inhibitory effects of these flavonoids on dihydropyrimidinase were substrate-dependent, dihydromyricetin significantly inhibited dihydropyrimidinase with IC_{50} values of 48 and 40 μM for the substrates dihydrouracil and 5-propyl-hydantoin, respectively. The results from the Lineweaver−Burk plot indicated that dihydromyricetin was a competitive inhibitor. Results from fluorescence quenching analysis indicated that dihydromyricetin could form a stable complex with dihydropyrimidinase with the K_d value of 22.6 μM. A structural study using PatchDock showed that dihydromyricetin was docked in the active site pocket of dihydropyrimidinase, which was consistent with the findings from kinetic and fluorescence studies. This study was the first to demonstrate that naturally occurring product dihydromyricetin inhibited dihydropyrimidinase, even more than the substrate analogs (>3 orders of magnitude). These flavonols, particularly myricetin, may serve as drug leads and dirty drugs (for multiple targets) for designing compounds that target several cyclic amidohydrolases.

四、攜回資料名稱及內容

The 67th Annual Meeting of the Japan Society for Cell Biology abstracts book.

科技部補助專題研究計畫出席國際學術會議心得報告

日期：105 年 10 月 11 日

計畫編號	MOST 103-2320-B-040-018-MY2		
計畫名稱	PriC 與 PriB 結合性質之研究		
出國人員 姓名	黃建智	服務機構 及職稱	中山醫學大學/生物醫學科學系/ 研究生(計畫兼任助理)
會議時間	105 年 6 月 7 日至 105 年 6 月 9 日	會議地點	日本/福岡
會議名稱	(中文)第 16 回日本蛋白質科學年會 (英文) The 16th Annual Meeting of the Protein Science Society of Japan		
發表題目	(中文)DnaD 的 C 端區域對單股 DNA 與 PriA 解旋酶的結合是重要的 (英文) The C-terminal region of DnaD protein is crucial for ssDNA and PriA helicase binding		

一、參加會議經過

The 16th Annual Meeting of the Protein Science Society of Japan was held from Jun 7 to 9, 2016 in Fukuoka, Japan. As the meeting is mainly based on biophysics for the main shaft, so lots of the participating researchers are in the fields of macromolecular interactions in which I am rather interested. In the lecture sections of Prof. Kenji Iwasaki, Prof. Takashi Fujii, and Prof. Ryo Nitta, I am deeply attracted to learn more about new and efficient strategies to develop my research scenario. I also contributed a post presentation (1P-032) at Day 1 (June 7). The title is “The C-terminal region of DnaD protein is crucial for ssDNA and PriA helicase binding.” Part of results for presentation in this conference has been published on PLoS One, 11, e0157593, 2016.

二、與會心得

I would briefly elucidate those summaries of my most impressive lectures as following:

1). The talk by Dr. Luo Fangjia, entitled “Processing of XFEL still images with a reference oscillation data set for crystal structural analyses of cytochrome c oxidase”, provides a method to analyze the structure.

In this method, serial femtosecond rotation crystallography (SF-ROX), a crystal mounted on a goniometer-head is exposed by the X-ray pulse and translates for the next exposure to obtain serial diffraction images with an equal rotation interval. To obtain high quality data set from SF-ROX diffraction images, data processing procedure was developed. A reference intensity data set is obtained by the oscillation method using crystals grown in the same batch as those used in SF-ROX method. Fix cell constant to those of SF-ROX data before processing. Crystal orientation parameters are refined to reduce the difference between I of the reference data and I/P of the target crystal, where P stands for partiality reflection. P is a function of the distance of a reciprocal lattice point from the reflection sphere. Once orientation parameters converge well in the refinement procedure, P is estimated and the observed intensity is evaluated by the formula,I/P.

2). The talk by Dr. Mariko Nagata, entitled "How do the RecJ/Cdc45 proteins work for the genome stability in Archaea?", in which they studied the genome stability of Archaea. RecJ/Cdc45-like sequence is conserved in the three domains of life. The bacterial RecJ protein plays important roles in a number of DNA repair and recombination pathways, whereas the cell division cycle 45 protein (Cdc45) is a member of the Cdc45-MCM-GINS (CMG) complex, working as a replicative helicase. The two RecJ-like proteins, RecJ1 and RecJ2, from *Thermococcus kodakarensis*, were characterized. Two RecJs showed different nuclease activity in vitro. RecJ1 formed complex with MCM and GINS, suggesting the possibility of the CMG-like formation. However, any nuclease activity of Cdc45 has not been reported to date, and therefore, the distance nuclease activity of RecJ1 suggests the different mechanisms for the replication fork progression between Archaea and Eukarya.

3). The talk by Dr. Eriko Aoki, entitled "Transmembrane domain of an adhesion is translocated into outer membrance without its signal sequence". The envelope of Gram-negative bacteria is composed of two distinct membranes, the inner membrane (IM) and the outer membrane (OM). The IM enclose the cytoplasm and is surrounded by the OM. These membranes are separated by the periplasmic space. Proteins embedded in OM are essential for passage of nutrients and wastes or virulence. These outer membrane proteins (OMPs) are synthesized in cytoplasm as precursor proteins with an N-terminal signal sequence. The signal sequence is believed to be required for the translocation across IM via Sec system. The signal sequence-less OMP is deficient in the ability to assemble into OM, and several OMPs expressed without signal sequence have been known to accumulate into inclusion bodies in cytoplasm. The transmembrane β-barrel domain of Haemophilus Influenzae adhesion (Hia) in *E.coli* was expressed and purified in high yield. It do not form inclusion bodies enough to be observed on SDS-PAGE with Coomassies Brilliant Blue staining. The sucrose gradient centrifugation experiment indicated that the transmembrane domain was found in OM as an assembled form.

4). The talk by Dr. Thomas P Halsted, entitled "The damage-free crystallographic study of denitrifying enzymes". During regular synchrotron radiation crystallography (SRX), protein metal centres undergo photoreduction and their ligand geometry is perturbed. Copper nitrite reductase (CuNiR) contains two copper centres, one T1Cu and one T2Cu, and catalyses the one electron reduction of nitrite to nitrous oxide. The T1Cu site acts to accept electron from either blue copper protein or c-type cytochromes. Complete electron transfer occurs from the T1Cu site only occurs when nitrite is bound at the T2Cu, suggesting a proton-coupled electron transfer (PCET) mechanism. This ordered mechanism is disputed however by kinetic studies, displaying evidence for a random mechanism. To fully elucidate the mechanism of CuNiR and investigate the nature of electron transport (ET) complex, high resolution native structures are required that collection at synchrotron sources. The collection of native CuNiR datasets, growing CuNiR-electron donor co-crystals and futures XFEL experiments to provide new insights into mechanism of CuNiR were discussed.

5). The talk by Dr. Ryuichiro Terada, entitled “Hybrid initio quantum mechanism analysis of O2-binding to the CuB-Fea3 binuclear center of cytochrome c oxidase”. Cytochrome c oxidase, which is the terminal enzyme of the electron transport chain, catalyzes an oxygen molecule to two water molecules, thus generating the driving force for proton pumps. This catalytic reaction (initiated from the reduce state) starts from binding of ligand (oxygen molecule) to the catalytic center of CcO, i.e., the binuclear center (BNC), which is composed of the CuB and heme a3 sites. To investigate the ligand recognition, the hybrid ab initio Quantum Mechanics (QM)/Molecular Mechanics (MM) Molecular Dynamics (MD) simulations was performed, which is one of the most advance computational molecular science techniques. As a result of the analysis, the binding of either O_2 or CO ligand to Fe of heme a3 induced the dynamical translocations of heme a3 was found, where the displacements were evolved within the heme plane. In the X-ray structures of CcOs in the complex with and without the ligands (CO and NO), the two-state static displacement of heme a3 were previously reported. The result from hybrid ab initio QM/MM MD simulations indicate that the anion acid residues in the vicinity of the BNC also modulate the configurations of the ligands, thus contributing to the catalytic reaction.

三、發表論文全文或摘要

ID: 1P-032

The C-terminal region of DnaD protein is crucial for ssDNA and PriA helicase binding

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Biomed. Sci., Chung Shan Med. Univ.

DnaD is a replication restart primosomal protein that reinitiates DNA replication in bacteria. In this study, we characterized the DNA-binding properties of DnaD from Staphylococcus aureus (SaDnaD) by using electrophoretic mobility shift analysis with a series of single-stranded DNA (ssDNA) homopolymers, fluorescence quenching, fluorescence resonance energy transfer assay, bioinformatic modeling tools, and three deletion mutant proteins, namely, SaDnaD1-195, SaDnaD1-200, and SaDnaD1-204. Protein–protein interaction within SaDnaD–SaPriA was also analyzed using surface plasmon resonance (SPR) and gold nanoparticle assays. SaDnaD forms distinct complexes with ssDNA of different lengths and stoichiometries (size of binding site) of 29 ± 2 nucleotides (nt) per tetramer. In fluorescence titrations, SaDnaD bound to ssDNA with a binding-site size of approximately 27 nt. The stable complex of SaDnaD1-195, SaDnaD1-200, and SaDnaD1-204 with ssDNA dT40 was not detectable, indicating that the C-terminal region of SaDnaD (especially aa 205–228) is significantly crucial for ssDNA binding. Results from SPR and gold nanoparticle assays revealed that SaDnaD1-195 cannot interact with SaPriA. In addition, SaDnaD1-195 did not stimulate the ATPase activity of SaPriA. Basing on these results, we conclude that the highly conserved C-terminal region of DnaD is crucial for both ssDNA and PriA helicase binding.

四、攜回資料名稱及內容

The 16th Annual Meeting of the Protein Science Society of Japan abstracts book.

科技部補助計畫衍生研發成果推廣資料表

日期:2016/10/11

科技部補助計畫	計畫名稱: PriC與PriB結合性質之研究
	計畫主持人: 黃晟洋
	計畫編號: 103-2320-B-040-018-MY2　　學門領域: 微生物及免疫學

無研發成果推廣資料

103年度專題研究計畫成果彙整表

計畫主持人：黃晟洋	**計畫編號：**103-2320-B-040-018-MY2
計畫名稱：PriC與PriB結合性質之研究	

成果項目					量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
國外	學術性論文	期刊論文			5	篇	1. Huang, C.C., Huang, C.Y.*(2016) DnaT is a PriC-binding protein. Biochem. Biophys. Res. Commun., 477, 988-992. (SCI) 2. Huang, Y.H., Lien, Y., Huang, C.C., Huang, C.Y.*(2016) Characterization of Staphylococcus aureus primosomal DnaD protein: Highly conserved C-terminal region is crucial for ssDNA and PriA helicase binding but not for DnaA protein-binding and self-tetramerization. PLoS One, 11, e0157593. (SCI) 3. Huang, C.Y.*(2015) Inhibition of a putative dihydropyrimidinase from Pseudomonas aeruginosa PAO1 by flavonoids and substrates of cyclic amidohydrolases. PLoS One, 10, e0127634. (SCI)

						4. Huang, Y.H., Huang, C.Y.*(2015) Creation of a putative third metal binding site in type II dihydroorotases significantly enhances enzyme activity. Protein Pept. Lett., 22, 1117-1122. (SCI) 5. Huang, Y.H., Huang, C.C., Chen, C.C., Yang, K.J., Huang, C.Y.*(2015) Inhibition of Staphylococcus aureus PriA helicase by flavonol kaempferol. Protein J., 34, 169-172. (SCI)
		研討會論文			2	1. Inhibition of dihydropyrimidinase by flavonoids and substrates of cyclic amidohydrolases, The 67th Annual Meeting of the Japan Society for Cell Biology 2. The C-terminal region of DnaD protein is crucial for ssDNA and PriA helicase binding, The 16th Annual Meeting of the Protein Science Society of Japan
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
參與計畫人力	本國籍	大專生			0	人次	
		碩士生			1		
		博士生			0		
		博士後研究員			0		
		專任助理			1		
	非本國籍	大專生			0		

		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）					

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
   　□實驗失敗
   　□因故實驗中斷
   　□其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   論文：■已發表　□未發表之文稿　□撰寫中　□無
   專利：□已獲得　□申請中　■無
   技轉：□已技轉　□洽談中　■無
   其他：（以200字為限）
   共發表5篇SCI論文,本人皆為通訊作者

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）
   這個計畫所帶出的研究成果除了基礎學術研究之外,並希望能進一步的提供新型抗生素的開發知識

4. 主要發現
   本研究具有政策應用參考價值：■否　□是，建議提供機關
   （勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
   本研究具影響公共利益之重大發現：■否　□是
   說明：（以150字為限）
   無