Project report

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人類輔促進因子 PC4 的結構，穩定度及動力之研究（II）

Studies of structure, stability and dynamics of human coactivator PC4 (II)

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Abstract

Human positive cofactor (PC4) containing a 127 amino acids with serine-rich regions near the N-terminus, is a general coactivator that enhances transcriptional activation of class II genes. PC4 interacts with a diverse array of proteins and DNAs and plays an important functional role in activator-dependent transcription during DA complex formation. Its coactivation function is medicated by phosphorylation. In order to better understand the functional role of PC4 in transcription activation and the process regarding the mediation of transcriptional regulation by phosphorylation and the structural basis of molecular recognition and interactions, the structure, stability and flexibility of unphosphorylated and phosphorylated PC4 needed to be studied. For structure studies, the first step is obtaining the pure and enough amount of PC4. In the first year's work of this grant, we were focusing on cloning, purification and characterization of recombinant PC4. In the second year's work, we were focusing on NMR data collection and chemical resonance assignments, three-dimensional structure determination and protein stability, and hydration studies.

PC4 cDNA was obtained from amplification by PCR from a HeLa cDNA library. The recombinant PC4 protein was obtained by growing *E. coli* bacteria harboring pET11a/PC4 in media or obtained by thrombin-cut GST-PC4 fusion protein expressed in *E. coli* bacteria with a vector (pGEX-2T) encoding a GST-PC4 fusion protein. Several functional and protein-protein interaction assays of recombinant PC4 protein were done in order to confirm that the recombinant proteins retain the function of native protein. These results indicate that recombinant PC4 was functionally equivalent to native PC4, both proteins markedly enhance GAL4-AH-activated transcription and can bind VP16 acidic activation domain to enhance activation. Various NMR data were collected and analyzed. Three-dimensional structures of protein were calculated from structure constraint data using structure-computing program such as distance geometry (DGII) (MSM, Insight II). Protein stability was studying upon a reversible denaturation of protein at extreme conditions of temperature and pH etc. NMR spectroscopy, circular dichroism were used to monitor the denaturation curves. High resolution solution structure determination, stability and hydration studies are continuing to be finished next year (grant (III)).

中文摘要:

人類輔促進子 PC4 由 127 個氨基酸所組成，其 N-端含有一段 serine-rich 區域，為常見之增強 class II genes 轉錄活性之輔促進因子。 PC4 能與很多種蛋白質及 DNAs 作用，並在需要促進子之轉錄作用過程中扮演重要的角色。它的活性並受磷酸化作用所調節。為了進一步了解 PC4 在轉錄活化所扮演的角色、磷酸化調節轉錄之機制及分子間相互辨識及交互作用之結構因子，研究未磷酸化及磷酸化 PC4 之結構、穩定度及可彎性是必需的。而研究結構，首先必需要有純度高且大量的 PC4。在這個計畫的第一個年度，我們致力於 recombinant PC4 cDNA 之選殖、蛋白質之純化及其特性分析。第二個年度，我們進行化學位移之確認、結構之計算、及穩定度、水合之研究等。明年將繼續完成之。

PC4 cDNA 由 HeLa cDNA library 利用 PCR 放大技術取得。Recombinant PC4 protein 由養殖帶有 pET11a/PC4 plasmid 之 *E. coli* 細菌，表達及純化得之，或由養殖含 pGEX-2T/GST-PC4 plasmid 之 *E. coli*，表達 fusion protein，再以 thrombin 切割 GST-PC4 fusion protein，分離及純化得之。 所得之 recombinant PC4 protein，再進一步做活性之確認及與它種蛋白質行交互作用之鑑定。Recombinant PC4 像 native PC4 一樣，具有加強 GAL-AH 活化轉錄作用之能力及與 VP16 acidic domain 接合、加強活化作用之特性。 Recombinant protein 確實具有與 native protein 相同之活性。

在各種不同 nmr 圖譜分析、確認各殘基之化學位移及取得距離及角度後，使用 distance geometry (如 DGII) 等電腦軟體來計算蛋白質之三度空間立體結構，並在不同之溫度及 pH 值及變性劑下，研究蛋白質之穩定度。 高解析度三度空間立體結構計算、穩定度、水合之研究，依進度，明年度將繼續完成 (grant (III))。

Background and specific aim

The expression of life in a living organism is built upon intricate communication network at various levels, ranging from DNA replication, transcription, expression to immune responses, cell proliferation and differentiation. The information flows during these biological events are mediated through direct, physical interactions between biomolecules. In order to understand these important biological events, detailed information at atomic-level concerning the structure, function, interactions of these biomolecules is prerequisite. The pivotal step is to obtain the three-dimensional structures and dynamics of these proteins, nucleic acids and their complexes at atomic resolution. A clear insight of the structure and dynamics of a protein will provide a solid framework for understanding of its functions, folding, stability and flexibility.

Human coactivator PC4, containing 127 amino acids with serine-rich region near its N-terminus, is a general coactivator that enhances transcription activation of class II genes. PC4 interacts with a diverse array of proteins and DNAs and plays an important functional role in activator-dependent transcription during DA complex formation. Its coactivation function is mediated by phosphorylation. The ability of PC4 to interact with a diverse array of proteins and DNAs will provide a great model to study the molecular recognition and interaction. Thus, we propose to study the structure, stability, hydration and dynamics of the phosphorylated and unphosphorylated forms of PC4 at first three years, and if possible, we will continue to study the PC4-DNA complex and PC4-protein interaction.

Completion of this study will lead us a better understanding the functional role of PC4 in transcription activation on the structural basis and the process regarding the mediation of transcriptional regulation by phosphorylation, and it will lay a foundation for understanding structure and function relationship and the mechanism underlining the molecular interactions and recognition during the key biological process on the structural basis.

Special aims:

The overall objective of this grant application is to investigate the structure, stability, hydration and dynamics of phosphorylated and unphosphorylated human PC4. Comparison of the structures of the phosphorylated and unphosphorylated human PC4 will provide additional information regarding the mediation of transcriptional regulation by phosphorylation. Completion of this grant study will lead us a better understanding the functional role of PC4 in transcription activation on

the structural basis and the process regarding the mediation of transcriptional regulation by phosphorylation, and it will lay a foundation for understanding structure and function relationship and the structural basis of molecular recognition and interactions.

The second year works are:

(1) Determination of the 3D structure of the human PC4 protein in solution (continued, 88/8-89/7)
(2) Study of the hydration of PC4 protein in aqueous solution (continued, 88/8~89/7)
(3) Study of the stability of PC4 by the effect of pH, temperature and denaturants. (continued, 88/8~89/7)

Results and discussions

Cloning of PC4 cDNA

Two oligonucleotides was synthesized: one is (5'-AATGCCTAAA TCAAATCAAAGGAACTTGTTTC-3') based on coding sequences (-1 to +26) in 5' end of the human PC4 cDNA and the other is (3'-TATCTACTGCGTCATTC TTTCGACATT-5') based on complementary sequences (+358 to +384) near the 3' terminus. These two oligonucleotides were used as primers and PC4 fragment was amplified by PCR from a HeLa cDNA library, a 385 bp fragment encoding human PC4 was obtained. The PC4 fragment was purified on a 1.2% agarose gel. The purified PC4 DNA fragment was then inserted into pGEX-2T vector (Amard) at SmaI restriction site and transformed into *E. coli* strain XA-90. The plasmid DNA with PC4 fragment was purified and isolated from transformed cells and was sent for sequencing to confirm the PC4 DNA sequences.

Expression and purification of recombinant PC4

Bacteria strain XA-90 was transformed with a plasmid encoding a GST-PC4 fusion protein. A fusion protein was expressed in bacteria by induction with IPTG. A 42 kDa glutathione S-transferase (GST)-PC4 fusion protein was obtained. Passing the lysate through glutathione-Sepharose beads and eluting with 15 mM glutathione as described by Ge and Roeder (1994) purified the fusion protein. The fusion protein was separated by thrombin digestion.

After thrombin cleavage of the glutathione S-transferase (GST)-PC4 fusion protein, intact recombinant PC4 was separated from GST by centrifugation. The

PC4 was in the supernatant. The supernatant was then applied to a single strand DNA Agarose column in order to remove thrombin. Bound PC4 was eluted from the column and the purity was checked by SDS-PAGE . The purity was around 98%.

To express the ^{15}N and ^{13}C double-labeled protein, the PC4 cDNA was inserted into pET11a expression vector and transformed into *E. coli* bacteria strain BL21. The uniformly ^{13}C, ^{15}N-labeled PC4 protein was obtained by growing *E. coli* bacteria harboring pET11a/PC4 in minimal media containing $^{15}N_4Cl$ and D-glucose-$^{13}C_6$. The recombinant ^{13}C, ^{15}N-labeled PC4 protein was purified by heparin-Sepharose column and phosphocellulose P11 column as described by Ge *et. al.* (1996).

Functional analysis of recombinant PC4 (rPC4)

Several functional assays and protein-protein interaction assay of recombinant PC4 protein were done in order to confirm that the recombinant proteins retain the function of native protein.

Native PC4 is able to markedly enhance GAL4-AH-activated transcription and can bind VP16 acidic activation domain to enhance activation. To confirm recombinant PC4 (rPC4) is functionally equivalent to native PC4, functional assay of increasing concentrations of free rPC4 in presence of GAL4-AH and VP16 on transcription from basal and activated templates. The data show a marked enhancement of GAL4-AH-activated transcription and a slight stimulation of basal transcription at low concentration of PC4 and then repression of basal transcription at higher concentration. Also, recombinant PC4 markedly enhanced activation by binding to the acidic activation domain of VP16. The protein-protein interaction assay of rPC4 and GST-VP16 fusion protein shows that rPC4 bound strongly to the GST-VP16 which is a good correlation between the capability of the VP16 activation domain to activate transcription and to bind PC4. These results indicate that recombinant PC4 is capable of substituting for native PC4 activity.

Acquisition and chemical resonance assignments of multi-dimensional homo- and hetero-nuclear NMR spectra

After the recombinant ^{13}C, ^{15}N-labeled PC4 protein was obtained. 2D homonuclear experiments (^1H-COSY, DF-COSY, 2Q-COSY, TOCSY, NOESY) and 2D heteronuclear experiments (^{15}N-HSQC, ^{13}C-HSQC) were acquired on a Bruker DPX 500 MHz NMR. 3D ^{13}C-HCCH-TOCY, ^{13}C-HCCH-COSY, ^{13}C and ^{15}N-NOESY-HSQC, HNCA, CBCA(CO)NH, HCA(CO)N spectra were acquired. All NMR data were processed on SGI computer using Bruker UXNMR program.

Measurements of $^3J_{HN\alpha}$, $^3J_{\alpha\beta}$, and $^3J_{N\beta}$ coupling constants and the stereo-specific assignments were done as described previously (Jeng et. al., 1994)

The chemical resonance have been assigned, and will be further confirmed.

Determination of the 3D structure of the human PC4 protein in solution

Structures of PC4 protein were calculated from the large number of available distance and torsion angle restraints derived from 2D and 3D NMR spectra. Initial structures were calculated by program DGII. Three-dimensional structure determination is continuing going, and will be finished next year.

Study of hydration of PC4

The hydration experiments are done partly by acquiring $^{13}C/^{15}N$-edited 2D and 3D NOESY and ROESY NMR spectra. The data are analyzed now.

Study of the stability of PC4 by the effect of pH, temperature and denaturants

The physical basis of stability of PC4 was obtained by studying of reversible denaturation curves at different temperatures, pH's and denaturants. Free energy for the underlying unfolding reactions will be calculated. The relative stability between phosphorylated and unphosphorylated forms will be calculated, too. The studies of the stability of PC4 will be continued and will be finished next year.

此研究成果將使我們從結構的觀點來了解磷酸化反應如何調控轉錄活化反應，並提供了解結構與功能關係所需之知識。

本計劃為三年才可完成之計畫，目前為第二年，大部份之研究還未至最後完成階段，明年將把最後成果做詳細之報告。

Reference:

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