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Expression, Purification and Spectral Characterization of p21$^{Waf1/Cip1}$

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Abstract p21$^{Waf1/Cip1}$, best known as a broad-specificity inhibitor of cyclin/cyclin-dependent kinase complexes, can interact with various target proteins, and this ability relies on its structural plasticity. Therefore, studies on the structural properties of p21 are very important to understand its structure-function relationship. However, detailed studies on its secondary structure and biophysical properties have been comparatively sparse. A human p21 gene was cloned into the temperature expression vector pBV220 and transformed into *Escherichia coli* strain JM109. Recombinant protein was expressed as a non-fusion protein and purified by gel filtration and anion exchange chromatography. The purified protein was verified by Western blot and the functional activity was recognized by pull-down assay. Furthermore, circular dichroism, fluorescence spectroscopy, and fluorescence quenching methods were used to characterize the conformational properties of the purified protein. The results indicate that it was largely unstructured under the native solution conditions, and its tryptophan residues were exposed and located in a positively charged microenvironment. This study lays a good foundation for further study of p21 binding to its different partners.

Keywords p21$^{Waf1/Cip1}$; PBV220; Circular dichroism; Tryptophan fluorescence

1 Introduction

Cell-cycle progression relies on not only the activation of cyclins, but also the activation of cyclin-dependent kinase(CDK), which successively acts together in G1 to initiate S phase and in G2 to initiate mitosis[1—3]. Since uncontrolled CDK activity is often the cause of human cancer, its function is tightly regulated by cell-cycle inhibitor. p21 is the first identified inhibitor of cyclin/CDK complexes, which regulates transitions between different phases of the cell cycle[4,5]. It is also known as Waf1[6], Cip1[7], Sdi1[8], Mda6[9], and Cap20[10] according to the different functions assigned to it by various groups since it has been cloned independently by a number of different routes.

Although many other CDK inhibitors have since been discovered[11], p21 appears to be the only inhibitor capable of interacting with essentially all of the CDK complexes. However, p21 targets are not limited to CDKs. p21 was found to bind many other regulatory proteins, such as PCNA, SAPK, ASK1 and CK2 kinases, Calmodulin and GADD45, procaspase 3, and oncogenic protein SET. And it is plausible that binding to the target proteins relies on the structural plasticity of p21. Therefore, studies on the conformational properties of p21 are very important to understand its structure-function relationship. However, detailed studies on its secondary structure and biophysical properties have been comparatively sparse.

In this study, lasmid pBV220 was used to construct expression vector pBV220-p21 in *E. coli* pBV220, which is a temperature-inducible system constructed by Zhang *et al.*[12], is not toxic to *E. coli* and is suitable for a large scale of expression. The recombinant protein was expressed with high efficiency by temperature and purified by gel filtration and ion exchange chromatography. Therefore, circular dichroism, fluorescence spectroscopy, and quenching methods were used to characterize the conformational properties of recombinant protein p21.

2 Experimental

2.1 Materials

The Q-Sepharose Fast Flow, Sephacryl s-100 and CNBr-actived Sepharose 4B were purchased from

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Amersham Pharmacia Biotech. Anti-p21(Ab-5) monoclonal antibody was purchased from NEO MARKERS (USA). Alkaline phosphatase anti-mouse IgG(H+L) was purchased from Beijing Dingguo Biotechnology (China). 5-Bromo-4-chloro-3-indolyl-phosphate(BCIP) and Nitro blue tetrazolium(NBT) were purchased from Sigma. The restriction endonucleases and T4 DNA ligase were purchased from TaKaRa. Primers were synthesized by Shanghai Sangon Biotechnology.

2.2 Plasmid Construction

The total RNA of p21 was isolated from human hepatocytes and the cDNA of p21 was cloned by RT-PCR. RT-PCR was generated with two specific primers: primer 1: 5'-AGCTGAATTCATGTCAGAA CCGGCTGGGGA-3' with engineered EcoRI restriction enzyme site; primer 2: 5'-TCGAGGATCCT TAGGGCTTCCTCTTGGACA-3' with engineered BamHI restriction enzyme site. Then the PCR product was double digested with EcoRI and BamHI, gel purified, and then ligated with doubly digested pBV220.

2.3 Expression, Purification and Identification of Recombinant Protein p21

Expression plasmid pBV220-p21 was transformed into *E. coli* strain JM109. Bacterial cells were grown at 30 °C in LB culture medium with 100 μg/mL ampicillin until A_{600} reached 0.6. Cell induction was initiated by enhancing the culture temperature to 42 °C, and cell growth was continued for another 6 h. The cells were harvested by centrifugation, resuspended in buffer A(20 mmol/L Tris, 1 mmol/L EDTA, 1 mmol/L β-ME, pH=7.5) containing 100 μg/mL lysozyme and sonicated. Inclusion bodies(IBs) were washed with buffer B(20 mmol/L Tris, 0.2 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L β-ME, pH=7.5) containing 0.5% Triton and buffer B containing 2 mol/L urea, respectively. IBs were resuspened in buffer B, heated at 100 °C for 5 min under constant shaking and cooled to 0 °C rapidly. After centrifugation, the supernatant was applied to a Sephacryl s-100 column, eluted with buffer A, and the elute containing p21 was collected. The elute containing p21 was pooled and then applied to a Q-Sepharose Fast Flow Column. The protein was eluted with a linear gradient NaCl buffer(from 0 mol/L to 1 mol/L NaCl). Western blot analysis was performed according to standard procedures[13]. For pull down assay, Calmodulin-(CNBr-activated) Sepharose 4B column was prepared according to the method described by Klee and Krinks[14]; the interaction of purified p21 and Calmodulin-Sepharose was designed according to the method reported before[15].

2.4 Circular Dichroism(CD) Spectroscopy and Fluorescence Spectroscopy

CD measurement was carried out on a Jasco J-810 spectropolarimeter. Fluorescence measurement was carried out on a JASCO FP-6500 spectrofluorometer. Fluorescence emission spectra were obtained by excitation at 295 nm. For fluorescence quenching, a fixed concentration of p21 was titrated with increased concentration of the quencher(acrylamide, KI, CsCl) and fluorescence emission intensity was monitored. For quenching studies with denatured p21, the protein was incubated with 6 mol/L urea overnight at r.t. The results were analyzed with the Stern-Volmer equation: $F_0/F=1+K_{sv}[M]$, where, F_0 and F are the fluorescence intensity at 350 nm in the absence and the presence of quencher. [M] is the concentration of quencher, K_{sv} is the Stern-Volmer constant for collisional quenching. The K_{sv} values of *N*-acetyltryptophanamide(Ac-Trp-NH_2) were chosen as a standard.

3 Results and Discussion

3.1 Expression, Prification and Ientification of Rcombinant Potein p21

As shown in Fig.1(A), a great amount of the recombinant protein was expressed after induction and visualized *via* SDS-PAGE at an expected apparent molecular mass of 21 KDa, which is in good agreement with the previous report[16]; and it was mainly located in the insoluble fraction of the cell as IBs(data not shown). It was also indicated that the prolonged induction time did not result in a significant increase of the expression yield of the recombinant proteins after induction for 6 h[Fig.1(A)]. For the purification of p21, several steps of washing were first carried out to get rid of the dissolved impurities from the IBs of p21 effectively[Fig.1(B), lanes 2 and 3]. The IBs of p21 could be easily dissolved by heating at 100 °C due to the advantage of the thermal stability of p21. Compared with using urea or Guanidine HCl to dissolve IBs of p21, heating denaturation can save time and does not require to get rid of urea or Guanidine HCl from the p21 solution. The dissolved IBs were