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FOR THE RECORD

Purification and crystallization of cyclin-dependent kinase inhibitor p21

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Abstract: p21, a universal inhibitor of mammalian cyclin-dependent kinases (CDK), regulates cell cycle progression by forming various distinct protein complexes with cyclins, CDKs, and the proliferating cell nuclear antigen. We have overexpressed recombinant human p21 in *E. coli* and purified active p21 to near homogeneity on a large scale. Crystals of recombinant p21 have been grown in the space group $P2_1$ a = 157.4, b = 152.7, c = 90.6 Å, and β = 92.7°. The diffraction data of the recombinant p21 have been collected to 2.5 and 3.5 Å resolution for the native crystal and two heavy atom derivatives of mercury and iridium.

Keywords: cell cycle; crystallization; cyclin dependent kinase inhibitor; X-ray diffraction

The primary control of eukaryotic cell cycle progression is provided by the sequential formation, activation, and subsequent inactivation of a family of serine/threonine protein kinases, consisting of a catalytic subunit, a CDK (*c*yclin-*d*ependent *k*inase), and an activating regulatory subunit, a cyclin. The enzymatic activity of a CDK is regulated by at least three different mechanisms: cyclin binding and activation, subunit phosphorylation, and association with and inhibition by a CDK inhibitor (reviewed by Sherr and Roberts, 1995). In mammalian cells, there exist at least two distinct families of CDK inhibitors, represented by two prototype CDK inhibitors, p21 and p16. The p16 family currently includes four isolated genes (reviewed by Sherr and Roberts, 1995): $p16^{INK4a}$ (also variously known as MTS1, *CDK4I*, and *CDKN2*), $p15^{INK4b}$ (also known as *MTS2*, $p15^{INK4b}$), $p18^{INK4c}$, and $p19^{INK4d}$ (reviewed by Sherr and Roberts, 1995). p16 family inhibitors regulate two closely related CDKs (CDK4 and CDK6) through two distinct mechanisms: 1) formation of binary complexes with the catalytic CDK4 or CDK6 to prevent activation by cyclin D binding and CAK phosphorylation (Serrano et al., 1993; Aprelikova et al., 1995), and 2) formation of inactive ternary p16-CDK4/6-cyclin D complexes (Hirai et al., 1995).

The p21 family currently includes three isolated genes, p21 (also known as *WAF1*, *Cip1*, *Sdi1*), $p27^{Kip1}$, and $p57^{Kip2}$ (reviewed by Sherr and Roberts, 1995). p21 was first discovered in normal human fibroblast cells as a component of a quaternary complex consisting of cyclin D, a CDK, p21, and the proliferating cell nuclear antigen [PCNA, (Xiong et al., 1992)], and later shown to be a component of most, if not all, cyclin-CDK complexes (Xiong et al., 1993b; Zhang et al., 1993). Newly synthesized p21 and PCNA protein was not detected in association with cyclin-CDK in cells transformed by a variety of tumor viruses or in p53-deficient Li-Fraumeni cells, suggesting that p53 function might regulate these higher order complexes (Xiong et al., 1993b). The p21 gene is transcriptionally activated by the tumor suppressor p53, and mice lacking p21 are defective in G1 checkpoint control (El-Deiry et al., 1993; Brugarolas et al., 1995; Deng et al., 1995; Waldman et al., 1995). In addition to p53 regulation, expression of the *p21* gene has also been found to be regulated by cellular senescence (Noda et al., 1994), the myogenic factor MyoD (Halevy et al., 1995), the antimitogenic growth factor TGF-b (Datto et al., 1995), vitamin D3 (Liu et al., 1996), and recently, CCAAT/enhancer-binding protein a [C/EBPa (Timchenko et al., 1996)], implicating the p21 gene in the regulation of a variety of growth control pathways.

p21 protein can regulate cell cycle progression by at least four distinct biochemical mechanisms: inactivating cyclin-CDK enzymes (Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993a), inhibiting PCNA-dependent DNA replication (Flores-Rozas et al., 1994; Waga et al., 1994), preventing phosphorylation and activation of CDKs by CAK (Aprelikova et al., 1995), and disrupting E2F-p107 and E2F-p130 from association with cyclin A-CDK2 (Zhu et al., 1995; Shiyanov et al., 1996). Such diverse biochemical functions of p21 largely result from the ability of this small protein to associate with several cellular proteins to form various distinct complexes, including binary complexes with CDKs, cyclins or PCNA, ternary complexes with a CDK and a cyclin, and quaternary complexes with a CDK, a cyclin, and PCNA. Deletion anal-

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Abbreviations: CDK, cyclin dependent kinase; CAK, CDK-activating kinase; PCNA, proliferating cell nuclear antigen.

ysis has identified two separate domains within the 164-amino acid human p21 coding sequence. Residues 1–71 alone are highly active in CDK binding and inhibition, and residues 141–160 efficiently bind PCNA and inhibit SV40 DNA replication in vitro (Chen et al., 1995; Luo et al., 1995; Nakanish et al., 1995). To provide a structural basis for the biochemical properties of p21, we have purified recombinant p21 on a large scale to near homogeneity. We report here a preliminary analysis of p21 crystal structure.

Purification and activity assay of p21

The p21 overexpression plasmid pET-p21 was constructed by insertion of the human p21 coding region into the vector pET-3d (Studier et al., 1990) and transformed into *E. coli* strain BL21(DE3). The transformed cells were grown in LB culture medium at 37 °C overnight. The p21 gene was overexpressed in the absence of isopropyl β-thiogalactopyranoside (IPTG). Addition of 0.5 mM IPTG did not significantly enhance the yield of the p21 protein. About 10 g of the prefrozen cell from a 2-liter culture was suspended in 40 mL of extraction buffer containing 20 mM Tris-base (pH 7.5), 1 mM EDTA, and 1 mM 2-mercaptoethanol. The suspension was passed through a French Press three times at 1,200 psi, and centrifuged at 27,000 × *g* on a Beckman J2-21M/E centrifuge at 4 °C for 20 min. The pellet was resuspended in 30 mL of 0.2 M NaCl, 20 mM Tris-base, 1 mM EDTA, and 1 mM 2-mercaptoethanol at pH 7.5. The suspension was heated at 100 °C for 5 min under constant shaking, immediately cooled in ice water, and centrifuged at 19,000 × *g* for 20 min at 4 °C. After 30 g of ammonium sulfate were slowly added into 100 mL of the supernatant, the mixture was stirred for 20 min and centrifuged at 19,000 × *g* for 20 min at 4 °C. The pellet was dissolved in about 15 mL of the extraction buffer and dialyzed against 1 liter of the extraction buffer twice (1 h each) at 4 °C with slow stirring. The supernatant was loaded onto a DEAE-Sepharose CL-6B column (2.5 × 25 cm, Sigma, St. Louis, MO) and eluted at 4 °C with a buffer of 0.2 M NaCl, 20 mM Tris-base,1 mM EDTA,1 mM 2-mercaptoethanol at pH 7.5 with a flow rate of 1 mL/min. p21 does not bind to DEAE in the presence of 0.2 M NaCl and passed through the column.

A typical batch of purification from 2 liters of bacterial culture yielded 10 to 20 mg of the p21 protein with greater than 90% purity as estimated by Coomassie blue staining . The recombinant p21 protein purified from *E.coli* retains its biological activity, as determined by the inhibition assay of the kinase activity of immunoprecipitated various cyclin-CDK enzymes (data not shown).

Two major technical difficulties were encountered during the large-scale purification of p21. First, p21 protein remained in the pellet after the cells were passed through a French press, indicating that the protein was in the form of an inclusion body. In order to solubilize the p21 protein, the pellet was resuspended in a buffer of 0.2 M NaCl, 20 mM Tris-base, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.5, and heated for 5 min at 100 °C. The gains from this step appeared to be twofold; not only was a large amount of p21 recovered from the inclusion body, but also many of the foreign proteins were removed from the sample upon their denaturation. The second problem with the purification of p21 was the phenomena of protein aggregation, as observed as follows: (1) Purified p21 could only be concentrated to approximately 2 mg/mL, while further concentration resulted in substantial loss of the protein. (2) When the p21 sample was loaded onto a Superose 6 column (Pharmacia), the elution profile showed several broad peaks corresponding to multiple aggregation states. (3) The native electrophoresis gel of p21 showed a smear of bands, again suggesting the multerization of the protein sample. (4) Laser light dynamic scattering could not determine the molecular weight of the purified p21, implying that there existed multiple magnitudes of p21 aggregation.

Although the purification described above normally yielded full-length p21, several batches resulted in the protein with a slightly higher mobility and a smaller molecular weight (about 1,000 daltons less) than full length p21, as seen on a denaturing SDS gel. Protein sequencing of the shorter p21 revealed an intact amino terminus, suggesting possible cleavage at the carboxyl terminal during purification. The precise carboxyl terminal sequence of the truncated p21 protein was not determined. The short form of p21 has an increase in solubility to about 7 mg/mL and appears to be less aggregated than full-length p21.

Crystallization and data collection

Two crystal forms were obtained from the truncated p21 under slightly different conditions. The hexagonal crystals were grown at room temperature by dialysis against a buffer containing 20 mM Tris-base, 0.5 mM NaN_3, 0.5 mM 2-mercaptoethanol, 0.5 mM EDTA, 6% polyethylene glycol 3350 (PEG 3350) at pH 8.5, while the monoclinic crystals were obtained from the same buffer plus 0.1 M NaCl. The heavy atom derivatives were prepared by soaking the native crystals at 25 °C in a buffer of 20 mM Tris-base at pH 8.5, 30% PEG 3350, and 10 mM K_3IrCl_6 for 2 weeks or 0.2 mM methyl mercury chloride for 3 days. The diffraction data of the native and the heavy atom derivatives were collected at room temperature in the phosphor image plate system Rigaku RAXIS, which was operated with an Rigaku RU200 X-ray generator and processed by the program DENZO.

The hexagonal crystals of the p21 protein are too small for the X-ray analysis. The p21 crystals in the monoclinic form have the space group $P2_1$ with cell dimensions of a = 157.4, b = 152.7, c = 90.6 Å, and β = 92.7 ° and a unit cell volume of 2,175,152 Å^3. If the calculated value of 18,130 daltons is used as the molecular weight of the p21 protein, the Vm values (volume of unit cell/molecular weight) will be 3.7 Å^3 per dalton for 16 molecules in the asymmetric unit, 2.5 for 24 molecules, and 2.0 for 30 molecules. This calculation suggests that the number of molecules in the asymmetric unit of the space group $P2_1$ may range from 16 to 30. However, considering the average Vm value of 2.4 Å^3/dalton for protein crystals and the observed hexagonal crystal form of p21, 24 molecules in an aggregation state of trimer or hexamer are likely to exist in the asymmetric unit of the monoclinic crystal form.

The monoclinic crystals diffract well to 2.5 Å resolution although the crystals were as small as 0.1 × 0.3 × 1.0 mm. The first native data was collected from freshly grown crystals with unit cell dimensions of a = 157.4, b = 152.7, c = 90.6 Å, and β = 92.7°. In order to circumvent the fact that the unit cell of the heavy atom derivatives were significantly reduced by 1 to 2% in all three dimensions upon soaking the native p21 crystals in the heavy atom stabilization solution, the second native data set was collected from crystals that were presoaked in a stabilization buffer (30% PEG 3350, 20 mM Tris-base at pH 8.5) for 3 days. The cell dimensions of the native p21 crystals were reduced to a = 156.0, b = 149.5, c = 89.0 Å, and β = 91.2° after soaking in 30% PEG buffer. Soaking the native and derivative crystals in the same 30% PEG stabilization buffer yielded similar cell dimensions and thus im-

Table 1. *Statistics for the diffraction data of p21*

Crystal	Resolution (Å)	Number of reflections Total	Unique	Complete (%)	R_{merge}[a]	R_{iso}[b]
Native1	2.5	193,962	89,895	62.4	0.076	
Native2	3.0	136,820	64,431	78.8	0.110	
Iridium	3.5	112,278	25,540	84.1	0.125	0.265
Mercury	3.2	107,704	50,783	75.2	0.097	0.320

[a] $R_{merge} = \sum_{hkl} \sum |I - \langle I \rangle| / \sum I$, the inner summation is over duplicated reflections, the outer is over unique reflections, and the denominator is summarized over all reflections.

[b] $R_{iso} = \sum_{hkl} F_{derivative} - F_{native}| / \sum_{hkl} F_{native}$.

proved the isomorphism between the native and derivatives. Statistics on the collection of the diffraction data are given in Table 1.

The crystals of the iridium derivative gradually turned to dark brown during 2 weeks of soaking, indicating good occupancy of the iridium atoms. The scaling R-factor (Riso in Table 1) and correlation coefficient were 0.348 and 0.465 for the mercury derivative, and 0.269 and 0.672 for the iridium derivative to 4.0 Å resolution. However, the correlation coefficients dramatically dropped to 0.324 and 0.488 at 3.5 Å resolution, indicating non-isomorphism at high resolution. Interpretatoion of difference Patterson synthesis is under way.

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