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Crystallization of a complex between human CDK6 and a virus-encoded cyclin is critically dependent on the addition of small charged organic molecules

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Human CDK6 plays an important role in controlling entry into the eukaryotic cell cycle. An activated complex of human CDK6 with a viral cyclin from herpesvirus saimiri was purified to homogeneity and crystallized using polyethylene glycol 3350 as precipitant. Crystallization was critically dependent on a narrow range of calcium acetate concentration and the presence of sulfo-betaine 201 as additive. Crystals belong to the hexagonal space group $P6_122$ or $P6_522$, with unit-cell parameters $a = b = 70.14$, $c = 448.77$ Å, $\gamma = 120^\circ$, and diffract X-rays to at least 3.1 Å resolution.

1. Introduction

Cyclin-dependent kinases (CDKs) control the progression through the eukaryotic cell cycle (Schafer, 1998; Sherr, 1994). The inactive apoenzyme is partially activated by complex formation with regulatory cyclin subunits. Complete activation requires additional phosphorylation of a threonine residue in the T-loop (Pavletich, 1999). Different CDKs are sequentially activated by different cyclin subunits during cell-cycle progression. Each successive cyclin–CDK complex phosphorylates a unique set of substrates that is essential for a given cell-cycle event. CDK4 and CDK6 form complexes with D-type cyclins that control the entry and G1/S progression in the eukaryotic cell cycle, where the commitment to complete the cell cycle is made (Sherr, 1994). Deregulation of the G1/S transition point is a common event in tumorigenesis (Hall & Peters, 1996; Sherr, 1996). Several viruses are known to encode in their genomes an ORF with cyclin homology and all known cyclin-encoding viruses can induce tissue hyperproliferation in infected hosts, which under certain circumstances can progress into clonal malignancies (Mittnacht & Boshoff, 2000). The two best characterized virus-encoded cyclins from herpesvirus saimiri (HVS; Jung *et al.*, 1994) and Kaposi's sarcoma herpesvirus (KSHV; Godden-Kent *et al.*, 1997; Li *et al.*, 1997) have the closest homology to human D-type cyclins and preferentially activate CDK6. CDK6 complexes with viral cyclins are resistant to cyclin-dependent inhibitory proteins (CDKI) of CIP/KIP and INK type and can phosphorylate retinoblastoma protein (pRB) as well as histone H1. Both viruses can induce malignant lymphoproliferations in certain hosts and the viral cyclins may contribute to the neoplasms associated with viral infections, in a similar way as overexpression of D-type cyclins contributes to cellular proliferation and transformation (Sherr, 1996).

In order to obtain a better understanding of the activation process of CDK6 by viral cyclins, we crystallized a complex of human CDK6 and virus-encoded cyclin from HVS (vcyclin). We report here the purification and crystallization of the complex and preliminary crystallographic characterization of the crystals.

2. Protein expression and purification

A bacterial expression plasmid with the gene for virus-encoded cyclin from HVS (pQE9-vcyclin) was obtained from J. U. Jung (Department of Microbiology and Molecular Genetics, Harvard Medical School) and a plasmid with the human CDK6 gene (pBSK-glob-CDK6) was obtained from M. Meyerson (Department of Pathology, Harvard Medical School). Recombinant baculoviruses were constructed for both genes using the Bac-to-Bac expression system (Life Technologies). The complete vcyclin gene was cloned into the pFastBac HTa donor plasmid between *Nco*I and *Hin*dIII restriction sites. The CDK6 gene encoding residues 1–308 was cloned into the pFastBac1 donor plasmid between *Bam*HI and *Hin*dIII restriction sites. Following standard procedures for the Bac-to-Bac system, recombinant baculovirus was obtained for both proteins. The recombinant vcyclin is expressed with an N-terminal His tag that can be cleaved by recombinant tobacco etch virus (rTEV) protease (Parks *et al.*, 1994). Sf9 cells were separately infected with the baculovirus constructs, resulting in about 8 mg l^{-1} soluble protein for vcyclin and 6 mg l^{-1} soluble protein for CDK6.

For purification of a CDK6–vcyclin complex, 0.5 l vcyclin-infected Sf9 cells and 0.75 l CDK6-infected Sf9 cells were lysed separately in

25 m*M* Tris–HCl pH 8.0, 10 m*M* NaCl, 1 m*M* PMSF, 8 µg ml^{-1} leupeptin, 4 µg ml^{-1} aprotinin using a Dounce homogenizer. After clearing the crude lysates by centrifugation at 3000*g*, the vcyclin and CDK6 supernatants were combined and centrifuged for 75 min at 300 000*g*. The high-speed supernatant was purified over a 7 ml iminodiacetic acid column loaded with Co^{2+} ions (Ostrove & Weiss, 1990). Bound CDK6 and vcyclin were co-eluted from the column in 20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl, 200 m*M* imidazole. Since only vcyclin has a His tag, co-elution of both proteins confirmed that CDK6 and vcyclin form a stable complex under purification conditions. The eluted complex was diluted 1:1(*v*/*v*) with 25 m*M* Tris–HCl pH 8.5. This sample was purified by anion-exchange chromatography over a 10 ml SourceQ column (Pharmacia) in 25 m*M* Tris pH 8.2, 25 m*M* NaCl, 0.5 m*M* EDTA, 2 m*M* DTT and eluted with an NaCl gradient from 25 to 500 m*M* NaCl in ten column volumes. The CDK6–vcyclin complex eluted at about 250 m*M* NaCl as a single sharp peak. To remove the N-terminal His tag from vcyclin, the pure protein eluted from the SourceQ column was diluted 1:1(*v*/*v*) with 50 m*M* Tris–HCl pH 8.0, 0.5 m*M* EDTA, 1 m*M* DTT and digested with rTEV protease (Life Technologies) at a ratio of 200 units of enzyme per milligram of CDK6–vcyclin complex for 3 h at 293 K. The His-tagged protease was removed with 2 ml Ni-NTA resin (Qiagen) by the batch method and the digested complex was finally purified over a SourceQ column under the same conditions as described above (Fig. 1). Pure CDK6 and vcyclin isolated by reverse-phase chromatography from the purified complex were analyzed on a Bruker electrospray iontrap mass spectrometer. The molecular mass calculated from the spectrum was 28 764 Da for vcyclin and 35 060 Da for CDK6, which is in good agreement with the theoretical values of 28 765 and 35 060 Da, respectively. Dynamic light-scattering analysis of the complex showed a polydispersity of 23%.

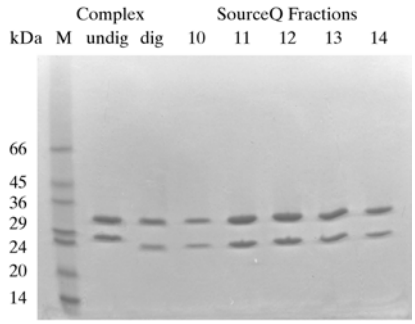

Figure 1
10–15% SDS–polyacrylamide gel electrophoresis of purified CDK6–vcyclin complex. The first two lanes show pure complex before and after rTEV digestion. The apparent molecular weight of CDK6 in the upper band is unchanged, while vcyclin after digestion shows the expected shift in molecular size from 31.7 to 28.7 kDa. Lanes 4–8 of the gel show the results of purifying the digested complex over a Source Q column. CDK6 and vcyclin co-elute from the column in fractions 10–14.

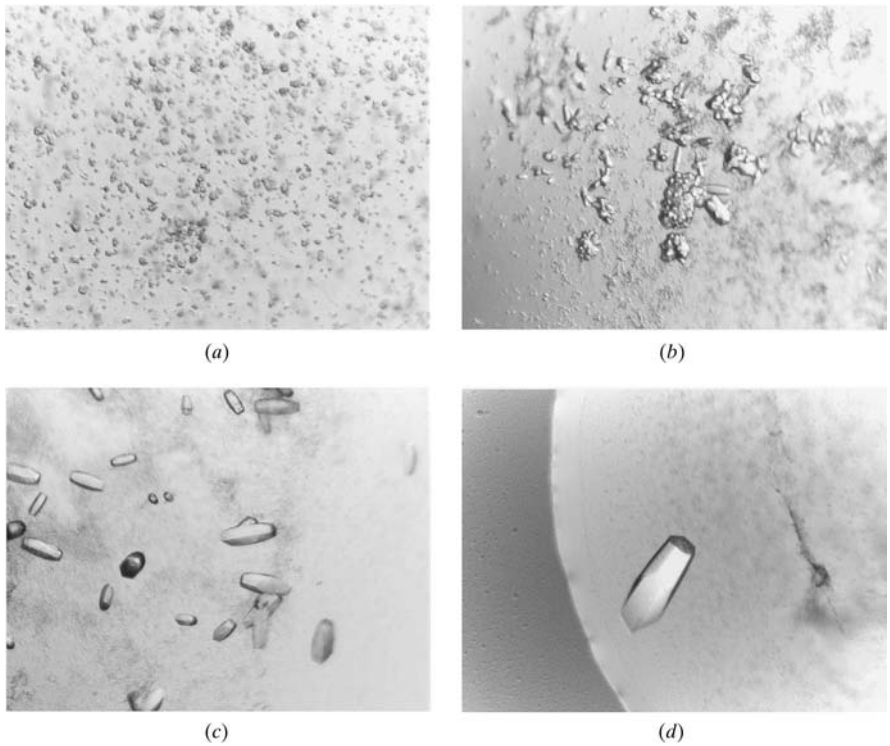

Figure 2
Photographs of CDK6–vcyclin complex crystals. (*a*) Globular crystalline aggregates grown from CDK6–vcyclin complex at 8 mg ml^{-1} with 1 m*M* inhibitor peptide GAGAKKSPKKAGAGA, 2 m*M* AMPPCP and 5 m*M* $MgCl_2$ in 10 m*M* Tris–HCl pH 8.0, 10% PEG 3350, 0.1 *M* sodium formate, 0.15 *M* NaCl. (*b*) Small crystals of the same protein/peptide mixture as in (*a*) grown in 10 m*M* Tris–HCl pH 8.0, 10% PEG 3350, 0.1 *M* calcium acetate, 0.15 *M* NaCl. (*c*) Hexagonal crystals of the same protein/peptide mixture as in (*a*) grown in 10 m*M* Tris–HCl pH 8.0, 10% PEG 3350, 0.1 *M* calcium acetate, 10 m*M* trimethylamine–HCl. (*d*) Protein crystals of CDK6–vcyclin alone at 10 mg ml^{-1} grown in 10 m*M* Tris–HCl pH 8.0, 10% PEG 3350, 0.1 *M* calcium acetate, 0.25 *M* sulfo-betaine 201. The average crystal size is 0.15 × 0.15 × 0.4 mm.

3. Crystallization

The complex was concentrated to 10 mg ml^{-1} in 25 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 10 m*M* DTT, 0.5 m*M* EDTA for crystallization experiments using the hanging-drop vapor-diffusion method. Crystallizations conditions were tested for CDK6–vcyclin complex alone and in solution with 2 m*M* adenylyl ($\beta\gamma$-methylen)-diphosphonate (AMPPCP), 5 m*M* $MgCl_2$ and 1 m*M* of a potential inhibitor peptide (GAGAKKSPKKAGAGA) that was found to inhibit CDK2 with an apparent K_m of 5.5 µ*M* (S. Pongor, personal communication). Initial screening was based on the sparse-matrix crystallization screening conditions developed by Jancarik & Kim (1991) and commercially available from Hampton Research. Results of the initial screens showed that precipitant concentrations in Hampton Crystal Screens I and II were too high and led to precipitation under most conditions. However, reducing precipitant concentrations to half revealed no promising crystallization conditions either. The best results were obtained with the polyethylene glycol (PEG)/Ion Screen (Hampton Research) at half concentration. Several conditions, including 0.1 *M* lithium nitrate, 0.1 *M* sodium formate, 0.1 *M* ammonium formate, 0.1 *M* magnesium acetate and 0.1 *M* calcium acetate with 10% PEG 3350 and 150 m*M* NaCl, resulted in small globular aggregates (Fig. 2*a*). Disturbing a crystallization setup made from 0.1 *M* calcium acetate, 0.15 *M* NaCl, 10% PEG 3350 with a hair led to the formation of small crystals (Fig. 2*b*) that were difficult to reproduce even after microseeding with seeds from the original crystals. Using this calcium acetate/PEG condition as a starting point, additives 11–20 from Additive Screen I and all conditions from Additive Screen II

Table 1
X-ray data-collection statistics.

Values in parentheses refer to the highest resolution shell

Resolution range (Å)	30.0–3.1
No. of observations	92567
No. of unique reflections	12678
Multiplicity	7.3
Completeness (%)	97.9 (81.8)
Average $I/\sigma(I)$	29.1 (4.0)
R_{sym}† (%)	5.8 (29.0)
Mosaicity	0.42

† $R_{sym} = \sum_{hkl} \sum_I |I_i - \langle I \rangle| / \langle I \rangle$, where I_i is the intensity of the ith measurement of reflection hkl and $\langle I \rangle$ is the average intensity of the reflection.

from Hampton Research were screened for their effect on crystallization of the CDK6–vcyclin complex without inhibitor peptide, AMPPCP and $MgCl_2$. Additives 14 and 16 from additive screen I and more than half of the additives from screen II promoted crystal growth. Crystallization conditions with 4% glucose, 0.04 *M* glycyl-glycyl-glycine, 0.01 *M* trimethylamine–HCl (Fig. 2*c*) and 0.28 *M* sulfo-betaine 201 (Fig. 2*d*) resulted in single well shaped crystals. Interestingly, the related sulfo-betaine 195 did not support any crystal growth. In the final optimized crystallization conditions, 0.7 µl CDK6–vcyclin complex in concentration buffer was combined with 0.7 µl 0.1 *M* calcium acetate, 10% PEG 3350 and 0.1 µl 2.0 *M* sulfo-betaine 201 and equilibrated against the precipitant 0.1 *M* calcium acetate, 10% PEG 3350, 0.15 *M* NaCl at 295 K. Under these conditions, single hexagonal crystals with average dimensions of 0.15 × 0.15 × 0.4 mm grew within 2 d.

4. Diffraction data collection

In order to find good cryoconditions for the complex crystals, three different cryoprotectants, PEG 400, PEG 3350 and glycerol, were tested in increasing concentrations in the mother liquor. The optimal conditions were 5–10 min soaks in mother liquor containing 0.28 *M* sulfo-betaine 201 and stepwise increasing concentrations of PEG 3350 from 10 to 30%. After a final soak in mother liquor with 0.28 *M* sulfo-betaine 201, 30% PEG 3350 and 5% glycerol, the crystal was mounted in a cryoloop, quickly cryocooled in liquid nitrogen and transferred to a cold stream at ∼100 K with cryotongs.

A complete data set from a crystal grown without AMPPCP and inhibitor peptide was collected at the Advanced Light Source (Lawrence Berkeley National Laboratory) beamline 5.02 on a Quantum 2 × 2 array CCD detector (Area Detector Systems Corporation). A total rotation range of 70° was collected with 1° rotation per frame at a distance of 300.14 mm and a wavelength of 0.9999 Å. Under these conditions, the crystals diffracted to at least 3.1 Å, the edge of the detector. Autoindexing and examination of the systematic absences in the data indicate that the space group is $P6_122$ or $P6_522$, with unit-cell parameters $a = b = 70.14$, $c = 448.77$ Å, $\gamma = 120°$. Assuming one CDK6–vcyclin complex in the asymmetric unit results in a Matthews coefficient (Matthews, 1968) of 2.5 Å^3 Da^{-1}, with 51% solvent content in the crystal. All data were processed with the *HKL* package (Otwinowski & Minor, 1997) and *TRUNCATE* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994), resulting in a complete data set of good quality to 3.1 Å (Table 1).

Using these data, we are in the process of solving the structure by molecular-replacement methods using the structures of vcyclin (Schulze-Gahmen *et al.*, 1999), CDK2–cyclinA complex (Jeffrey *et al.*, 1995) and CDK6–p19^{INK4d} complex (Brotherton *et al.*, 1998) as models.

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