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Overexpression and Spectroscopic Characterization of a Recombinant Human Tumor Suppressor $p16^{INK4}$

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$p16^{INK4}$, which is a 16-kDa polypeptide protein, inhibits the catalytic activity of the CDK4-cyclinD complex to suppress tumor growth. Both unlabeled and isotope-labeled human tumor suppressor $p16^{INK4}$ protein were overexpressed and purified to characterize biochemical and structural properties. The purified p16 binds to monomeric GST-CDK4 and exists in a monomer conformation for several weeks at 4°C. The circular dichroism (CD) data indicates that p16 contains high percentage of α-helix and that the helix percentage maximized at pH value of 7.0. One- and two-dimensional nuclear magnetic resonance (NMR) data suggest that purified p16 from our construct has a unique folded conformation under our experimental conditions and exhibits quite stable conformational characteristics.

Keywords: $p16^{INK4}$, Circular dichronism, CDK4 inhibitor, NMR, Tumor suppressor.

Introduction

The cell cycle of eukaryotic cells is regulated by a family of protein kinases called the cyclin-dependent kinases (CDKs) (Morgan, 1995; Nigg, 1995). The sequential activation of different CDKs following phosphorylation of their substrates promotes progression of the cell cycle. During G1 phase, it was previously demonstrated that complexes of CDK4 and cyclinD control cell proliferation (Connel-Crowley *et al.*, 1993; Sherr, 1993; Lees, 1995).

It has been reported that $p16^{INK4}$ as a negative regulator of cell-cycle progression inhibits the catalytic activity of CDK4-cyclinD enzyme by binding to CDK4 (Aprelokova *et al.*, 1995; Hirai *et al.*, 1995; Parry *et al.*, 1995; Xiong *et al.*, 1996). Recently, Koh *et al.* demonstrated functional defects in several tumor-derived p16 alleles and suggested a strong correlation between p16 activities and tumor susceptibility. Since a number of groups (Kamb *et al.*, 1994; Mori *et al.*, 1994; Kyritsis *et al.*, 1996; Zuo *et al.* 1996) have proven that p16 protein may be involved in the development of many different tumors such as melanoma, lung adenocarcinoma and esophageal cancer, etc. Thus p16 could be a good candidate to suppress tumor growth for anticancer therapy (Nobori *et al.*, 1994; Koh *et al.*, 1995; Wolfel *et al.*, 1995).

Very recently, high-resolution NMR and circular dichroism studies for different constructs of refolded $p16^{INK4}$ have been reported (Tevelev *et al.*, 1996). However, since the refolded p16 proteins were quite unstable and exhibited dynamical behavior in structure, it was not possible to determine three-dimensional structures in solution. Despite the molecular and biological importance of the protein, not much information about $p16^{INK4}$ molecule from biochemical or spectroscopic studies is available. Therefore, we present the biochemical and spectroscopic characterization of native human $p16^{INK4}$ together with detailed overexpression and purification procedures to aid further structural studies such as NMR and X-ray crystallography.

Materials and Methods

Plasmid construction Human wild type p16 full length c-DNA was amplified using PCR from human placenta c-DNA library (Serrano *et al.*, 1993). For the convenience of subcloning, we introduced a NdeI restriction enzyme site in 5′ primer (5′-GGAGATCTCATATGATGGAGCCTTCGGCTGACTG-3′) and a Bam H1 site in 3′ primer (5′-GGGGATCCTCAATCG-GGGATGTCTGAGGGACC-3′) respectively. PCR amplified p16 c-DNA was sequenced to confirm mutation-free and subcloned into NdeI-Bam H1 site of pET15b bacterial expression vector. In addition to the intact p16 protein, the expressed recombinant protein will have extra 22 amino acids at its N-terminus coded

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from the vector sequence (Histidine tagged region, thrombin cutting site, see pET15b vector sequence from Novagen catalog).

Overexpression and purification For overexpression of $p16^{INK4}$, *E. coli* strain BL21 was transformed with pET-p16 and grown at 37°C in M9 medium [0.5% (W/V throughout) D-glucose, 0.1% NH_4Cl, 0.05% NaCl, 0.6% Na_2HPO_4, 1 mM $MgSO_4$, pH 7.4] supplemented with 0.001% D-biotin and thiamine, 0.4% casemino acid and 100 μg/ml ampicillin. IPTG 1 mM solution was treated to induce the production of p16 protein when optical density (OD_{600}) reached 0.8. After 2 h of induction, the cells were harvested by centrifugation for 20 min and stored at −70°C prior to purification. For ^{15}N-labeling of protein, M9 minimal medium containing $^{15}NH_4Cl$ from Isotec Inc. (Miamisburg, U.S.A.) as a sole nitrogen source was used for cell growing.

All purification steps were carried out at 4°C. The frozen cells were suspended in 3/80(v/v) of original culture volume using buffer A (20 mM Tris-HCl pH 7.9, 0.5 M NaCl, 1 mM PMSF) containing 0.1% NP-40. The suspended cells were lysed by sonication on ice using a Branson Sonifier Model 450 sonicator and cell debris was removed by ultracentrifugation for 20 min. The supernatant was loaded onto Histidine-binding column equilibrated with buffer A containing 5 mM imidazole. The column was washed with buffer A containing 5 mM imidazole and with buffer A containing 20 mM imidazole, 40 mM imidazole and 60 mM imidazole. The absorbed protein was eluted using buffer A containing 200 mM imidazole and dialyzed using dialysis membrane in 25 mM sodium phosphate buffer at pH 7.0 containing 500 mM NaCl, 1 mM PMSF, 1 mM EDTA and 1 mM DTT. The dialyzed protein was concentrated using centricon-3 concentrator. Final yield was approximately 8 mg/L and the purity was determined by 15% SDS-PAGE and ^1H-1D NMR spectra.

CD and NMR experiments CD data were collected on a Jasco J-715 spectropolarimeter using 1.0 mm path length cells. Scan speed was 500 mM/min, 1 nm band width and the total number of accumulation was 32. The samples were prepared as three 8 mM volume in H_2O solution for pH values of 6.0, 7.0, 8.0, and 9.0. A standard noise reduction procedure was used for final spectrum.

The protein sample for NMR experiments consisted of 0.3~0.7 mM uniformly ^{15}N-enriched p16 in 90% H_2O/10% ^2H_2O, pH 7.0, 500 mM NaCl, 25 mM sodium phosphate, 1 mM EDTA and 1 mM DTT at temperatures of 25°C and 30°C. One-dimensional (1D) proton and two-dimensional (2D) ^1H-^{15}N correlated sensitivity enhanced pulsed-field-gradient heteronuclear single quantum coherence (HSQC) (Kay *et al.*, 1992) spectra were collected on a Bruker DMX600 spectrometer equipped with a gradient unit and an actively shielded triple resonance probehead.

Results and Discussion

Purified p16 protein was judged as a purity of >95% by SDS-PAGE. A single SDS-PAGE band of purified product is shown in Fig. 1. A native gel-electrophoresis result of a single band (data not shown) and ^{15}N resonance linewidths from ^1H-^{15}N 2D-HSQC spectrum suggest the purified p16 is a predominantly monomer conformation in our purified condition. The monomer conformation in our experimental condition was also confirmed by data from gel filteration HPLC elution profile comparing molecules of standard molecular weights. The elution profile suggests the molecular weight of purified p16 in our experimental condition is close to that of Equine myoglobin (M.W. 17 kDa) and the protein does not form any oligomers in our experimental condition (Fig. 2).

The purified recombinant p16 made a complex with purified CDK4 and showed inhibition of CDK4-cyclinD1

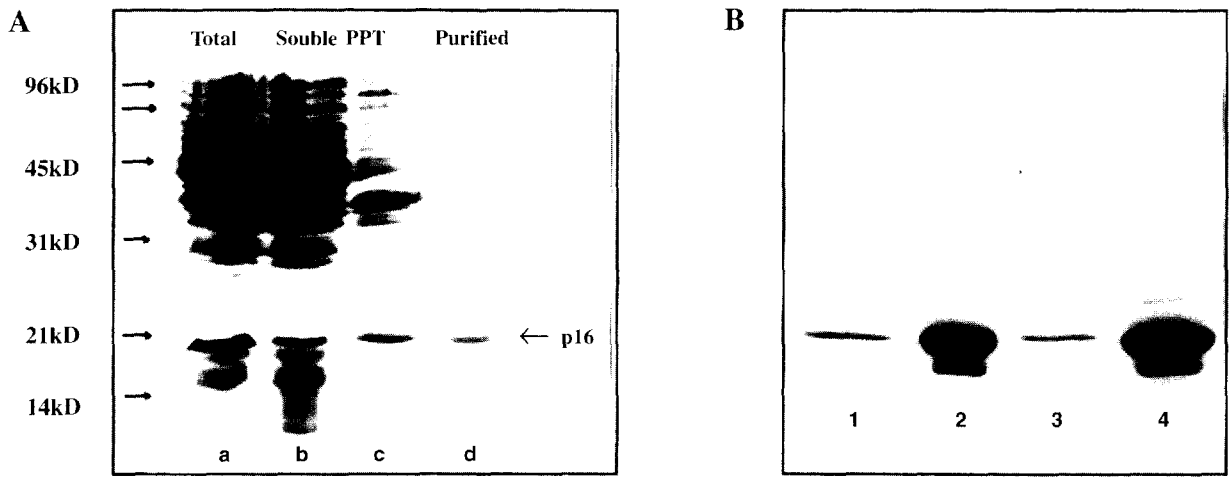

Fig. 1. SDS-PAGE of $p16^{INK4}$ with 15% gel. (A) Lane a, total extract after 2 h of IPTG induction at 37°C; lane b, soluble extract before column loading; lane c, precipitated fraction after sonication; lane d, purified p16 fraction by the procedure described in the text. (B). SDS-PAGE without boiling with protein kept at 4°C. Lane 1, 4 wk with 1 mM DTT and concentration of 80 μg/ml; lane 2, 4 wk with 1 mM DTT and 8 mg/ml concentration; lane 3, 2 wk with 100 mM DTT and 80 μg/ml concentration; lane 4, 4 wk with 100 mM DTT and protein concentration of 8 mg/ml.

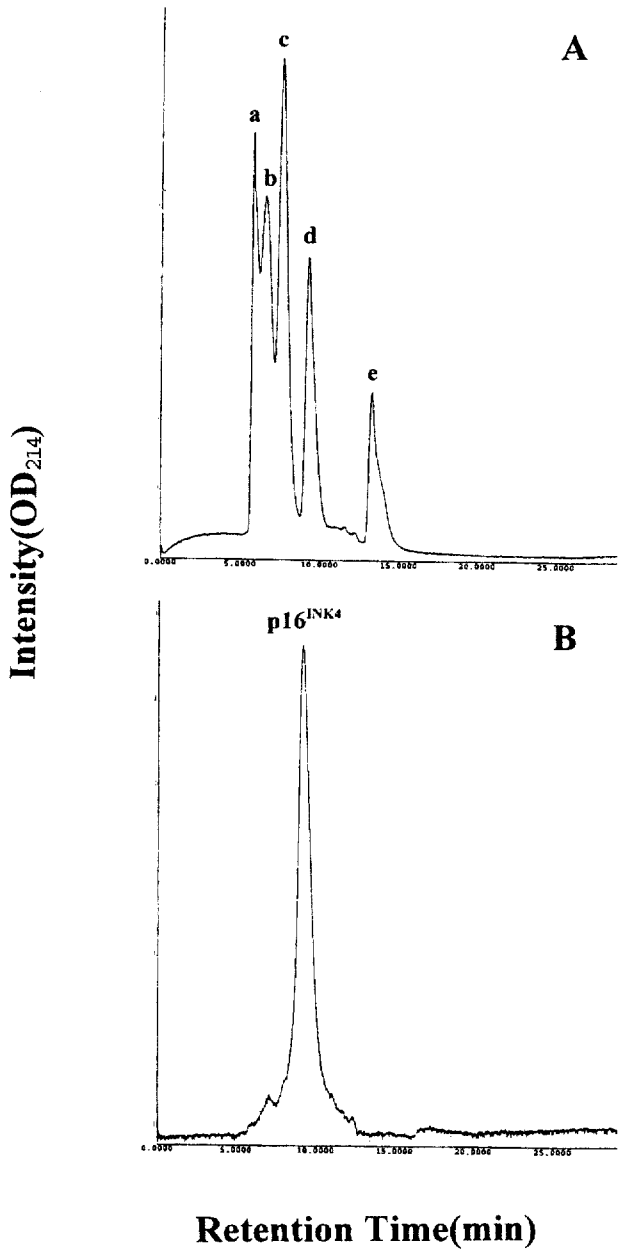

Fig. 2. Gel filteration HPLC elution profile. (A) Reference proteins were loaded to determine retention times depending on molecular weight. (a) Tyroglobulin (M.W. 670 kD) (b) Bovine gamma globulin (M.W. 158 kD) (c) Chicken ovalbumin (M.W. 44 kD) (d) Equine myoglobin (17 kD) (e) Vitamine B-12 (1.35 kD). (B) A single purified p16 elution profile showing that retention time is close to that of Equine myoglobin (17 kD).

activity in the enzyme assay (data not shown) as demonstrated in literature (Wolfel *et al.*, 1995). These results prove that our purified p16 is functionally active.

In order to observe conformational properties, CD measurement was performed at four pH values. The CD spectrum show that the intensity of negative ellipticity at 222 nm does not change at pH values 6, 8, and 9. The profile of pH 9.0 is very close to that of pH 6.0. Figure 3 shows the results from CD spectrum at pH values of 7, 8, and 9. CD data suggests that major secondary structural elements are α-helices from the negative ellipticity at 222 nm. Since the positive ellipticity is not observed at 234 nm, it is expected that there is no detectable β-sheet conformations in the p16 protein. This was confirmed by one-dimensional proton NMR and 2D ^{1}H-^{15}N HSQC spectra (Figs. 4 and 5). It is interesting to see the subtle change of negative ellipticity at pH value of 7. The negative intensity at 222 nm does not change much from pH 8.0 to 9.0. However, the intensity of negative ellipticity at 222 nm increased about 20% at pH 7.0. Thereby it is expected that the α-helical percentage shifts at pH value of 8.0 and helices are also stable at pH 7.0.

After 1 day of NMR data collection at 30°C, protein solution had a small amount (~10% from 1D NMR analysis) of precipitation in sample tube and the sample of 0.7 mM concentration exhibited a slow process of degradation during 4 days of data collection at 25°C. The p16 protein was also prepared having up to 1 mM concentration. However, when the protein sample is higher than 0.8 mM, it aggregated in one day at 25°C in NMR sample tube. Tevelev *et al.* (1996) observed severe aggregations and oligomerizations at 0.2 mM concentration in refolded p16 protein having eight N-terminal amino acids deleted. However, we could not find any oligomerizations in our NMR sample in the period of 4 wk (Fig. 1B) and it was also observed that p16 is quite stable at 4°C of temperature for several weeks, showing no indication of precipitation or degradation procedure. Thereby it is expected that p16 protein from our construct would be quite stable even in high concentrations with native form.

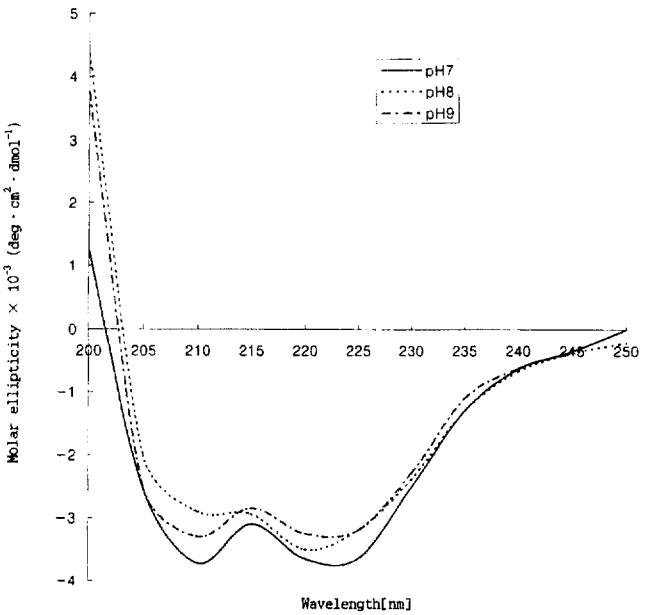

Fig. 3. CD spectrum of $p16^{INK4}$ at different pH values. Baseline was corrected by the buffer soluion for each pH value.

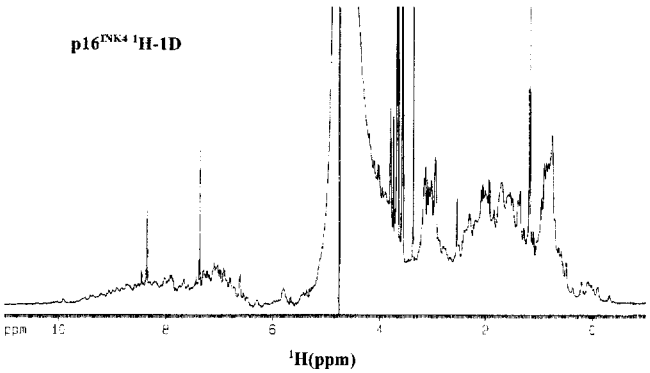

Fig. 4. One-dimensional ^1H NMR spectrum of soluble p16^{INK4} in 90% H_2O/10% ^2H_2O solution collected on a Bruker DMX600 spectrometer at pH 7.0, 25°C. Presaturation of water was applied to minimize water signal.

Figure 4 shows one-dimensional ^1H NMR spectrum of p16 in soluble form in 90% H_2O/10% ^2H_2O solution at pH 7.0. To monitor concentration dependence of p16, two 1D ^1H spectrum were collected at protein concentrations of 0.3 mM and 0.7 mM. A number of high-field shifted methyl proton resonances ranged from −0.5 to 1.0 ppm strongly suggests that the protein has a defined tertiary structure at this experimental condition. Since no spectral and linewidth change was observed for different concentrations, it is expected to maintain native conformation at range of 0.3~0.7 mM concentration. In order to confirm homogeneity of protein, ^{15}N-correlated 2D HSQC spectra (Fig. 5) was collected. The spectra also shows that p16 protein under NMR experimental conditions retains a unique and folded conformation from a number of cross peaks at low field area of proton chemical shifts. Some of assigned amino acids involved in secondary structures are labeled in Fig. 5. In addition, it is expected that the percentage of α-helix is dominant based on resonances ranged from 7.5 to 9.0 of backbone amide protons.

Because it is still unclear how p16 protein interacts with CDK4, this report will help structure-function studies by providing a structural background and high purity (>90%) of stable p16 for structural analysis. As p16 protein contains four ankyrin regions, it could be expected that p16 will be involved in protein–protein interaction with CDK4 protein and/or cyclinD. Several groups also reported that INK4 family (Guan *et al.*, 1994; Hannon & Beach, 1994; Kamb *et al.*, 1994; Chan *et al.*, 1995) which are p16, p15, p18, and p19, inhibits the activity of cyclin-CDK complexes and shares structural similarity for interaction with CDKs. Since not much is known about how these inhibitors control the regulation of CDK complexes specifically, the biochemical as well as three-dimensional structural information will serve as an important step to understand p16 regulations of cyclinD-CDK4 complex during G1 phase of cell cycle. For this purpose, three-dimensional structural studies of both free p16^{INK4} and

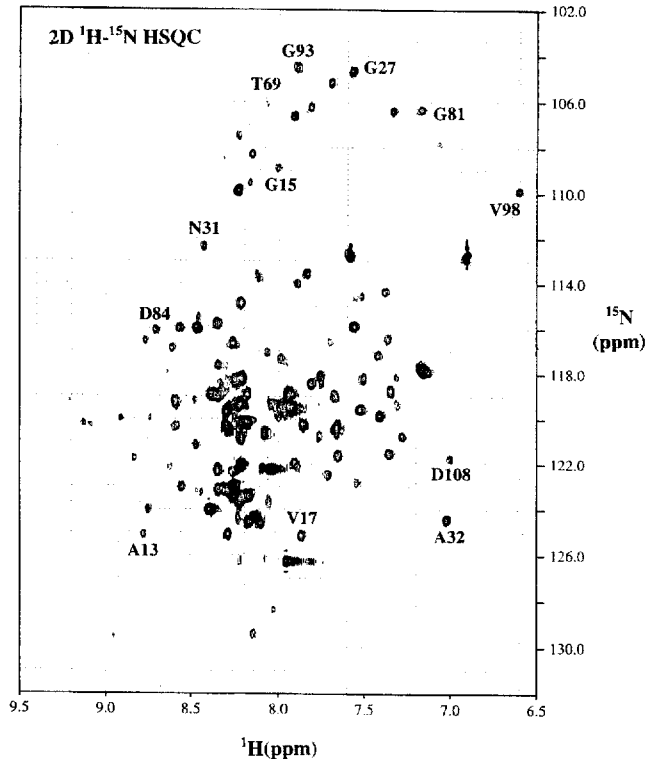

Fig. 5. ^1H-^{15}N 2D-HSQC spectrum of p16.^{INK4} A Pulsed-Field-Gradient (PFG) technique was used for water suppression and the suppression of artifacts. Garp (Shaka *et al.*, 1985) sequence was used for ^{15}N decoupling during the acqusition period. Data set was collected as a 256 (^{15}N) × 1024 (^1H) complex matrix.

CDK4/p16 complex based on heteronuclear multi-dimensional NMR data are in progress.

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References

Aprelikova, O., Xiong, Y., and Liu, E. T. (1995) Both p16 and p21 families of cyclin-dependent kinase (CDK) inhibitors block the phosphorylation of cyclin-dependent kinases by the CDK-activating kinase. *J. Biol. Chem.* **270**, 18195–18197.

Chan, F. K. M., Zhang, J., Cheng, L., Shapiro, D. N., and Winoto, A. (1995) Identification of human and mouse p19, a novel CDK4 and CDK6 inhibitor with homology to p16^{INK4}. *Mol. Cell Biol.* **15**, 2682–2688.

Connel-Crowley L., Solomon, M. J., Wei, N., and Harper, J. W. (1993) Phosphorylation independent activation of human cyclin-dependent kinase 2 by cyclin A *in vitro*. *Mol. Biol. Cell* **4**, 79–92.

Guan, K-L, Jenkins, C. W., Li, Y., Nichols, M. A., Wu, X., O'keefe, C. L., Matera, A. G., and Xiong, Y. (1994) Growth suppression by p18, a p16$^{INK4/MTS1}$- and p14$^{INK4B/MTS2}$-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes Dev.* **8**, 2939–2952.

Hannon, G. J. and Beach, D. (1994) $p15^{INK4B}$ is a potential effector of TGF-beta-induced cell cycle arrest *Nature* **371**, 257–261.

Hirai, H., Roussel, M. F., Kato, J. Y., Ashmun, R. A., and Sherr, C. J. (1995) Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. *Mol. Cell. Biol.*, **15**, 2672–2681.

Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavitgian, S. V., Stockert, E., Day, R. S., Johnson, B. E., and Skolnick, M. H. (1994) A cell cycle regulator potentially involved in genesis of many tumor types. *Science* **264**, 436–440.

Kay, L. E., Keifer, P., and Saarinen, T. (1992) Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity. *J. Am. Chem. Soc.* **114**, 10663–10665.

Koh, J., Enders, G. H., Dynlacht, B. D., and Harlow, Ed (1995) Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. *Nature* **375**, 506–510.

Kyritsis, A. P., Zhang, B., Zhang, W., Xiao, M., Takeshima, H., Bondy, M. L., Cunningham, J. E., Levin, V. A., and Bruner, J. (1996) Mutations of the p16 gene in gliomas. *Oncogene* **12**, 63–67.

Lees, E. (1995) Cyclin dependent kinase regulation. *Current Opinion in Cell Biology* **7**, 773–780.

Morgan, D.O. (1995) Principles of CDK regulation. *Nature* **374**, 131–143.

Mori, T., Miura, K., Aoki, T., Nishihira, T., Shozo, N., and Nakamura, Y. (1994) Frequent somatic mutation of the MTS1/CDK4I (multiple tumor suppressor/cyclin-dependent kinase 4 inhibitor) gene in esophageal squamous cell carcinoma. *Cancer Res.* **54**, 3396–3397.

Nigg, E. A. (1995) Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. *Bioessays* **17**, 471–480.

Nobori, T., Miura, Wu, D. J., Lois, D., Takabayashi, K., and Carson, D. A. (1994) Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* **368**, 753–756.

Parry, D. Bates, S. Mann, D.J., and Peters, G. (1995) Lack of cyclin D-Cdk complexes in Rb-negative cells correlates with high levels of $p16^{INK4/MTS1}$ tumour suppressor gene product. *EMBO J.* **14**, 503–511.

Serrano, M., Hannon, G. J., and Beach, D. (1993) A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* **366**, 704–707.

Shaka, A. J., Barker, P. B., and Freeman, R. J. (1985) Computer-optimized decoupling scheme for wideband applications and low level operation. *J. Magn. Reson.* **64**, 547–552.

Sherr, C. J. (1993) Mammalian G1 cyclins. *Cell*, **73**, 1059–1065.

Tevelev, A., Byeon, I-J., Selby, T., Ericson, K., Kim, H-J., Kraynov, V., and Tsai, M-D. (1996) Tumor suppressor $p16^{INK4A}$: structural characterization of wild-type and mutant proteins by NMR and circular dichroism. *Biochemistry* **35**, 9475–9487.

Wolfel, T., Hauer, M., Schneider, J., Serrano, M., Wolfel, C., Klehmann-Hieb, E., Plaen, E. D., Hankeln, T., Buschenfelde, K.-H., and Beach, D. (1995) $p16^{INK4A}$-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. *Science* **269**, 1281–1284.

Xiong, Y., Kuppuswamy, D., Li, Y., Livanos, E. M., Hixon, M., White, A., Beach D., and Tlsty, T. D. (1996) Alteration of cell cycle kinase complexes in human papillomavirus E6- and E7-expressing fibroblasts precedes neoplastic transformation. *J. Virol.* **70**, 999–1008.

Zuo, L., Weger, J., Yang, Q., Goldstein, A. M., Tucker, M. A., Walker, G. J., Hayward, N., and Dracolopi, N. C. (1996) Germline mutations in the $p16^{INK4A}$ binding domain of CDK4 in familial melanoma. *Nature Genet.* **12**, 97–99.