Expression and characterization of Syrian golden hamster p16, a homologue of human tumor suppressor *p16^{INK4A}*

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Abstract

The *p16^{INK4A}/CDKN2A* tumor suppressor gene is known to be inactivated in up to 98% of human pancreatic cancer specimens and represents a potential target for novel therapeutic intervention. Chemically induced pancreatic tumors in Syrian golden hamsters have been demonstrated to share many morphologic and biological similarities with human pancreatic tumors and this model may be appropriate for studying therapies targeting *p16^{INK4A}/CDKN2A*. The purpose of this study was to investigate the fundamental biochemistry of hamster P16 protein. Using both in vivo and in vitro approaches, the CDK4 binding affinity, kinase inhibitory activity, and thermodynamic stability of hamster and human P16 proteins were evaluated. Furthermore, a structural model of hamster P16 protein was generated. These studies demonstrate that hamster P16 protein is biochemically indistinguishable from human P16 protein. From a biochemical perspective, these data strongly support the study of *p16*-related pancreatic oncogenesis and cancer therapies in the hamster model.

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As a negative regulator of the key protein kinases, cyclin-dependent kinases 4 and 6 (CDK4 and 6), the protein product of *p16 (INK4A/CDKN2A)* is involved in cell cycle control regulation through inhibition of CDK-mediated phosphorylation of the retinoblastoma gene product (Rb). Phosphorylation of Rb results in the release of active transcription factor, E2F, from Rb-E2F transcription factor complexes and, subsequently, triggers the activation of a number of genes that are required for G0-to-G1 and G1-to-S transitions [1]. Genetic alterations of *p16* have been found in numerous cancer cell lines and primary tumors including melanomas, leukemias, lymphomas, glioblastomas, and cancers of the esophagus, lung, bladder, colon, breast, and head and neck [2]. In human pancreatic carcinomas, *p16* al-

terations have been reported in as many as 98% of specimens [3]. Several studies confirm that potentially inactivating genetic events result in loss of *p16* expression in human pancreatic cancers [4,5]. A significantly increased risk of pancreatic cancer in melanoma-prone kindred families with germline *p16* mutations lends further support to the role of *p16* inactivation in human pancreatic carcinogenesis [6]. Finally, transfection of wild-type *p16* gene into human and mouse pancreatic cancer cell lines leads to cell arrest [7].

Pancreatic cancer is the fifth most common cancer causing death in the United States [8] and five-year survival rates have been reported to be 4% [9]. Currently, surgical resection remains the only hope for long-term survival, although attempts at surgical resection are possible in only 10–20% of cases due to the presence of advanced disease at the time of presentation. Clearly, there is an urgent need for the development of novel

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therapeutic strategies and this has been the focus of an NIH investigation [10].

Chemically induced pancreatic tumors in the Syrian Golden hamster (*Mesocricetus auratus*, SGH) are a well-established model for evaluation of the development and progression of pancreatic adenocarcinoma. These tumors share many striking morphologic and biological similarities with human pancreatic tumors [11,12]. *N*-Nitroso-bis-(2-oxopropyl) amine (BOP)-induced SGH pancreatic tumors are the only experimental model that demonstrates a ductal morphology, which is typical of human pancreatic cancers [12,13]. Hamster pancreatic tumors also demonstrate many biological similarities with human tumors including the propensity for aggressive local invasion, metastases, and cachexia. Similar genetic manifestations include early *Ha-ras* alterations [14], amplification of *c-erbB2* and *EGFR* [15], increased activation of telomerase [16], alterations in the *p53* tumor suppressor gene [17], and inactivation of the *p16^{INK4A}/CDKN2A* gene [18]. Of note, hamster pancreatic tumors can be easily induced at high frequencies by certain chemicals (e.g., BOP or DMBA) and with relatively short tumor latencies. All of these factors suggest that the hamster model may provide a unique prospect to determine the comparative step-wise progression of genetic alterations in human and hamster pancreatic oncogenesis. Based on this information, it is reasonable to utilize the hamster model in order to investigate the possible involvement of *p16* inactivation in hamster pancreatic tumors and to explore the potential application of *p16*-targeted cancer therapy. Previous reports from our laboratory indicate that *p16* inactivation is a common event in tumorigenic hamster cell lines [19] and in BOP-induced SGH pancreatic tumors [18]. It is necessary, however, to investigate the fundamental biochemistry of hamster P16 protein, prior to the formulation of strategies targeting *p16* for prevention and therapy, in this model.

Here we present a study on the function, structure, and stability of hamster P16 protein as compared to those of human P16 protein. The results demonstrate that hamster P16 protein binds to human CDK4 in vitro and in vivo with an affinity comparable to that of human P16 protein, and that both proteins exhibit almost identical CDK4-inhibitory activities. Structurally, hamster P16 protein displays a thermodynamically unfavorable conformation similar to that of human P16 protein.

Materials and methods

Cloning, expression, and purification of hamster *p16*. Total RNA from Syrian Golden hamster pancreas was isolated using TRIzol Reagent (Invitrogen) with two sequential rounds of purification as recommended by the manufacturer. After treatment with DNase I

(Ambion) to remove residual genomic DNA, 1.0 µg of total RNA was used as template for first strand synthesis using the PowerScript reverse transcriptase kit (BD Clontech). The first strand synthesis reaction mixture was then treated with RNase H (Amersham USB) to remove the RNA hybrid. Subsequently, 2.0 µL of this mixture was used as template in RT-PCR using the following primers: 5'-GAT GCT CTG AAT TCA TGG AGC CCT CTG CGG ACG-3' (forward) and 5'-GAC ATC TTC TCG AGT CAG TAG GGC CCT AGG GGG TG-3' (reverse). The resultant RT-PCR product was cloned into a pGEX-6p-1 vector at *EcoRI* and *NotI* restriction sites (Amersham Pharmacia), and the DNA sequence of the clone was determined.

The recombinant pGEX-6p-hamster *p16* plasmid was transformed into *Escherichia coli* BL21 (DE3) pLys S cells (Novagene), and hamster *p16* was expressed as a GST-fusion protein (glutathione-S-transferase) upon IPTG induction. For GST-tagged hamster P16 protein, the lysate from bacteria harboring the pGEX-6p-hamster *p16* plasmid was purified on a reduced glutathione-agarose column (Sigma) equilibrated with 20 mM Tris, 1 mM EDTA, and 0.15 M NaCl (pH 7.4 at room temperature). After elution with reduced glutathione, GST-hamster P16 was further purified by a Q Fastflow column (Pharmacia). To purify hamster P16 protein without a GST tag, 100 units of PreScission protease (Amersham Pharmacia) was added to the GST-hamster P16 in PBS buffer. After incubation at 4°C for 24 h, the protein solution was loaded onto a reduced glutathione-agarose column equilibrated with 20 mM Tris, 1 mM EDTA, 0.15 M NaCl, and 5 µM β-mercaptoethanol (pH 7.4 at room temperature). The flowthrough was concentrated and further purified by a S100 column (Pharmacia) equilibrated with 5 mM Hepes, 1 mM EDTA, and 1 mM DTT (pH 7.4 at room temperature). The final product was analyzed by Western blot using an anti-human P16 monoclonal antibody (BD PharMingen).

Expression and purification of other proteins. Human *p16* cDNA was cloned and expressed as a GST-fusion protein in BL21 (DE3) pLys S as previously described [20]. The purification of GST-fused human P16 and free human P16 was similar to that of hamster P16 except that the GST tag was removed with 100 units of Thrombin (Sigma).

The human CDK4/cyclin D2 holoenzyme was expressed and purified as follows [21]. Briefly, human *CDK4* cDNA, obtained by RT-PCR from total RNA of HeLa cells, was subcloned into a pBacBAK8 phagemid (BD Clontech) to generate the pBAC/CDK4 transfer vector, in which a Hisx6 tag was fused to the C-terminus of CDK4. The cDNA for human *cyclin D2* was cloned by PCR into *EcoRI* and *BglII* restriction sites of pBacPAK8 vector to yield a pBAC/cyclin D2 transfer vector. Baculovirus was constructed using *Autographa californica* nuclear polyhedrosis virus BacPAK6/Bsu 361 DNA and *Spartanoptera frugiperda* Sf-9 cells following the manufacturer's instructions (BD Clontech). Both baculovirus particles, each containing the above transfer vectors, were co-transfected into HighFive cells (Invitrogen) and the CDK4/cyclin D2 complex was purified by modifying a published procedure [22]. Lysate from transfected insect cells was centrifuged and the supernatant was loaded onto a TALON (BD Clontech) resin column equilibrated with a washing buffer, 20 mM Tris-HCl (pH 7.4 at room temperature), 100 mM NaCl, 0.1 mM Na₃PO₄, 1 mM NaF, 10 mM β-glycerophosphate, 5 mM β-mercaptoethanol, 0.2 mM AEBSF, 5 mg/mL aprotinin, and 5 mg/mL leupeptin. This was followed by washing using the same washing buffer plus 10 mM imidazole. Finally, the column was eluted with the washing buffer containing 50 mM imidazole. The eluent was dialyzed against kinase buffer containing 50 mM Hepes (pH 7.5 at room temperature), 10 mM MgCl₂, 2.5 mM EGTA, 0.1 mM Na₃VO₄, 1 mM NaF, 10 mM β-glycerophosphate, and 1 mM DTT, and further concentrated to approximately 0.3 mg/mL. Aliquots were stored at -80°C.

In vitro CDK4 activity assay. The in vitro CDK4 activity assay involved 3 units of the CDK4/cyclin D2 complex (~0.3 µg of protein) and varying concentrations of P16 protein in the kinase buffer (50 mM Hepes, pH 7.5, 10 mM MgCl₂, 2.5 mM EGTA, 0.1 mM Na₃VO₄, 1 mM NaF, 10 mM β-glycerophosphate, and 1 mM DTT) supplemented with 0.2 mM AEBSF, 2.5 mg/mL leupeptin, and 2.5 mg/mL aprotinin in a

total volume of 15.0 μ L [21]. One unit of CDK4 kinase is defined as the amount of CDK4 that catalyzes the incorporation of 1 μ mole of phosphate into Rb at 30°C within 15 min. These reagents were pre-incubated for 30 min at 30°C. Subsequently, GST-Rb379-928 (50 ng) and 5 μ Ci [γ - 32 P]ATP were added and the reaction mixtures were incubated for another 15 min at 30°C. After SDS-PAGE, the CDK4 activity was determined by quantifying the incorporation of 32 P into Rb using a PhosphorImager (Molecular Dynamics). Measurements were repeated in triplicate.

The substrate of CDK4, GST-Rb379-928, was prepared as follows. Human “large pocket” Rb cDNA, corresponding to residues 379–928 of wild-type Rb, was cloned into pGEX-2T plasmid and expressed as a GST-fusion protein in *E. coli* BL21 (DE3) pLys S. The cell lysate was purified on a glutathione-agarose column; the bound protein was eluted with 50 mM reduced glutathione in PBS buffer and then dialyzed against the kinase buffer as described above.

Pull-down assay. To investigate the interaction between N-terminal GST-tagged hamster or human P16 proteins and the human CDK4/cyclin D2 complex, 10 μ g of the CDK4/cyclin D2 complex and 20 μ g of GST-tagged hamster or human P16 proteins were incubated at 4°C in 250 μ L PBS (pH 7.4) for 2 h. The concentrations of CDK4-cyclin D2 and hamster or human P16 were 0.4 and 2.0 μ M, respectively. Then 250 μ L of freshly prepared reduced glutathione-agarose beads (equilibrated with PBS buffer at 4°C) was added to the reaction mixture and the incubation was continued for another 1 h with gentle agitation. The reaction mixture was loaded onto a spin column (Fisher Scientific) and centrifuged at room temperature, 1500g for 3 min. Subsequently, the column was washed with 1.0 mL PBS, and eluted with 80 μ L PBS buffer containing 20 mg/mL of reduced glutathione (Sigma) [23]. The eluent was further analyzed with Western blot using anti-human CDK4 antibody (sc-260, Santa Cruz). To investigate the competition between human and hamster P16 proteins, 1.0 mL of the reaction mixture including 0.1 μ M CDK4-cyclin D2, 0.5 μ M GST-hamster P16, and varying amounts of free human P16 or 0.1 μ M CDK4-cyclin D2, 0.5 μ M GST-human P16, and varying amounts of free hamster P16 in PBS (pH 7.4) was incubated at 4°C for another 2 h. The agarose-bound proteins were analyzed by Western blot as described above.

Yeast two-hybrid analysis. The MATCHMAKER LexA two-hybrid system (BD Clontech) was used in this study. Hamster p16 cDNA was cloned into the activation domain fusion plasmid, pB42AD, at *Eco*RI and *Not*I restriction sites. Human CDK4 cDNA was cloned into the binding domain fusion plasmid, pLexA, as previously described [23]. In this assay, protein/protein binding was determined by the activation of a LexA-dependent *lacZ* reporter, and the strength of the interactions was quantified in a liquid β -galactosidase activity assay. All transformation, expression, and β -galactosidase activity liquid

experiments were performed according to the manufacturer's instructions. The yeast strain EGY48, harboring p8op-*lacZ*, was used as the host.

Guanidinium chloride titration and circular dichroism analysis. Lyophilized proteins were dissolved in borate buffer (20 mM sodium borate and 40 μ M dithioerythritol, pH 7.5) and dialyzed overnight twice against 2 L of the borate buffer. A stock solution of $\sim$ 8.5 M guanidinium chloride was prepared in the same borate buffer. Samples containing 7.5 μ M proteins were incubated with different amounts of guanidinium chloride (the final concentrations ranged from 0 to 7.0 M) on ice overnight and then equilibrated at 20°C just prior to circular dichroism (CD) analysis [24]. Far-UV CD spectra (190–260 nm) were recorded on a JASCO J-500C spectropolarimeter using a quartz microcell of 0.1 cm light path length, and the exact concentrations of guanidinium chloride were calculated using its refraction index. In this study, the ellipticity at 222 nm, which is an indicator of the existence of α -helical secondary structure, was taken as the measure of the degree of structure present in the protein at each denaturant concentration. The concentration of guanidinium chloride at half-denaturation and the free energy of denaturation in aqueous condition were obtained on the basis of two-state approximation [25].

Sequence alignment and structural modeling. Amino acid sequence alignment was performed using the software Anthept [21]. The Pfam program was used to localize ankyrin repeats [23]. SWISS-MODEL was used for structural modeling by using the NMR solution structure of human P16 (PDB Code: 1A5E) as template [20].

Results and discussions

Cloning, expression, and Western blot of hamster p16 cDNA

Hamster p16 cDNA, derived from normal Syrian Golden hamster tissue, encodes a protein of 158 amino acid residues. As shown in Fig. 1, most residues of hamster P16 protein are conserved with respect to human and other rodent P16 proteins, particularly the ankyrin repeat regions. Overall, hamster P16 protein shares 70%, 76%, and 80% identity with human, mouse, and rat P16 proteins, respectively. In the “core” region (containing 4 ankyrin repeats), hamster P16 protein shares 81% identity (87% similarity) with human P16.

Fig. 1. Amino acid sequence alignment and the secondary structure analysis of P16 proteins. Anthept software was used to do the alignment, while the ankyrin repeats were determined using Pfam program. Residues with different degrees of identity are shown in different colors: 100%, red; 50–75%, blue; and less than 50%, black. The red lines represent the ankyrin repeats.

Fig. 2. Western blot of P16 proteins. After purification and removal of GST tag, 5.0 μ g of recombinant hamster and human P16 proteins was loaded on a 10% SDS–PAGE gel and blotted against anti-human P16 monoclonal antibody (Cat. No. 13251, PharMingen). Lane 1, pre-stained protein marker (Bio-Rad); lane 2, recombinant human P16; and lane 3, recombinant hamster P16.

DNA sequencing of multiple hamster genomic DNA and cDNA clones from different hamster tissues and cell lines (data not shown) demonstrated that residue 116 in hamster P16 is an Arg amino acid, rather than previously published Cys (GenBank Accession No. AF292567) [19].

To date, no anti-hamster P16 antibody is commercially available; therefore, free hamster P16 protein was blotted with an anti-human P16 monoclonal antibody (Cat. No. 13251, BD PharMingen). Strikingly, hamster P16 protein reacts positively and strongly against anti-human P16 antibody (Fig. 2). This finding illustrates the similarity between hamster and human P16 proteins from an immunohistochemical perspective, but also provides an extremely useful tool to detect hamster P16 protein in future studies.

Hamster p16 interacts with human CDK4 in vitro and in vivo

The interaction between hamster P16 and human CDK4/cyclin D2 holoenzyme, using pull-down assays, was first examined to establish the inhibitory function of hamster P16 protein. Human CDK4-cyclin D2 holoen-

zyme was used because the holoenzyme, not free CDK4, is the biological active component [23]. As shown in lane 6 of Fig. 3, CDK4 was detected in the pull-down product (eluent), suggesting that hamster P16 bound to the CDK4-cyclin D2 complex. A positive control with human P16 protein was performed, as shown in lane 2. Moreover, when both human and hamster P16 proteins existed in the same pull-down reaction mixture, they competed with each other for CDK4 binding. When increasing amounts of free hamster P16 protein were added to the GST-human P16/CDK4-cyclin D2 mixture, there was a decreasing amount of CDK4 present in the pull-down product. When the molar ratio of free hamster P16:GST-human P16 was 2:1, CDK4 was detected in the pull-down product (lane 3). As the ratio increased to 5:1, less CDK4 was present in the pull-down product (lane 4). When free hamster P16 was in great excess (hamster:human = 10:1) (lane 5), no CDK4 was detected in the pull-down product of GST-human P16/CDK4-cyclin D2. Similar observations were obtained when free human P16 was used as a competitor in a GST-hamster P16/CDK4-cyclin D2 reaction. As the molar ratio of free human P16 and GST-hamster P16 proteins increased from 0:1 to 5:1, the amount of CDK4 detected in the pull-down product decreased (lanes 2, 7, and 8). When this ratio increased to 10:1 (free human P16:GST-hamster P16), no CDK4 was detected in the pull-down product. The affinities of both human and hamster P16 proteins appear “equivalent” in this assay

Fig. 3. Pull-down assay to probe the interactions between P16 proteins and the CDK4-cyclin D2 holoenzyme in vitro. Reaction mixtures containing GST-tagged P16 proteins and CDK4-cyclin D2 in the absence or presence of competitor proteins were incubated with reduced glutathione-agarose. After elution with free reduced glutathione, bound proteins were blotted with anti-human CDK4 antibody (sc-260, Santa Cruz). Lane 1, the input containing 5% of the amount of CDK4-cyclin D2 in other lanes; lanes 2, 3, 4, and 5, GST-human P16 (0.5 μ M)/CDK4-cyclin D2 (0.1 μ M) containing 0, 1.0, 2.5, and 5.0 μ M of free hamster P16, respectively; lanes 6, 7, 8, and 9, GST-hamster P16 (0.5 μ M)/CDK4-cyclin D2 (0.1 μ M) containing 0, 1.0, 2.5, and 5.0 μ M of free human P16, respectively; and lane 10, BSA, bovine serum albumin, as negative control. HU, human; HA, hamster.

Fig. 4. Yeast two-hybrid analysis of the interactions between P16 and human CDK4 *in vivo*. Protein/protein interactions were assessed colorimetrically by the liquid galactosidase enzymatic activity assay as described by the manufacturer. For each protein/protein pair, the first protein was fused to the LexA DNA binding domain while the second protein was fused to the activation domain, AD. Lam was provided by the manufacturer as a negative control. Assays were performed in triplicate.

since either protein can block the CDK4-binding of the other one in the molar ratio of 10:1 (free:GST-tagged).

In order to confirm the above *in vitro* results, the yeast two-hybrid assay was employed to study the interaction between hamster P16 and human CDK4 *in vivo*. Both human P16 and P18 were used as positive controls, and Yar 1, a yeast protein containing three ankyrin repeats, was used as negative control [23,26]. As shown in Fig. 4, human P16 and P18, and hamster P16 all bound to human CDK4, while no detectable interaction was observed between Yar 1 and human CDK4. The results suggest that hamster P16 bound to human CDK4 *in vivo* with specific affinity. It appears that the rank order of human CDK4-binding strength was: human P16 $\geq$ hamster P16 > human P18.

Hamster p16 inhibits human CDK4 kinase activity

An *in vitro* assay was developed in order to evaluate the kinase activity of CDK4 in response to hamster P16 protein. As the concentration of hamster P16 increased in the reaction mixture, the activity of CDK4 kinase decreased accordingly (Fig. 5). The IC_{50} value, or the concentration of hamster P16 protein required to achieve 50% of the maximum inhibitory activity, is 85 ± 20 nM, and this is comparable to human P16 kinase inhibitory activity (60 ± 20 nM) [20]. This quantitative finding strongly suggests that the biological function of hamster P16 protein is similar to that of human P16.

Fig. 5. *In vitro* kinase assay to determine the CDK4-inhibitory activities of P16 proteins. Each reaction mixture included 3 units of CDK4-cyclin D2, 50 ng GST-Rb379-p28, 5 μ Ci [γ - 32 P]ATP, and various amounts of P16 proteins. After reaction at 30 °C for 15 min, the reaction mixtures were separated by 10% SDS-PAGE and the incorporation of γ - 32 P into Rb protein was quantified using a PhosphorImager (Molecular Dynamics). (●) Represents hamster P16 and (○) represents human P16 protein. All measurements were performed in triplicate.

Conformational stability of hamster and human p16 proteins is similar

Protein stability is one of the key factors affecting function. The conformational stability of hamster P16 protein was evaluated in a guanidinium chloride-induced protein denaturation study [27]. Fig. 6 shows the CD denaturation curves of ellipticity at 222 nm versus

Fig. 6. Guanidinium hydrochloride-induced denaturation curves of P16 proteins at 20 °C monitored by far-UV CD. Samples containing 7.5 μ M proteins were incubated with different amounts of guanidinium chloride on ice overnight and analyzed by far-UV CD (190–260 nm) at 20 °C. The ellipticity at 222 nm was plotted against the concentration of guanidinium hydrochloride to obtain those parameters evaluating the conformational stability of P16 proteins. (●) Represents hamster P16 and (○) represents human P16.

Table 1
Conformational stability of P16 proteins^a

Protein	$D_{1/2}$ (M)	$\Delta G_d^{\text{water}}$ (kcal mol ⁻¹)	m (kcal mol ⁻¹ M ⁻¹)
Human P16	0.60	1.94	3.23
Hamster P16	0.75	2.21	2.94

^a $\Delta G_d^{\text{water}}$, $D_{1/2}$, and m values were calculated according to a two-state model [24], and the estimated error in such analyses is $\pm 5\%$.

guanidinium chloride concentrations. The values of $\Delta G_d^{\text{water}}$ and slope m are listed in Table 1. Hamster P16 protein behaved very similar to human P16 in terms of its $D_{1/2}$, $\Delta G_d^{\text{water}}$, and m values, suggesting little difference in the conformational stability between both proteins. The CD experiments also indicated that the free energy of unfolding was relatively small for both proteins (2.21 and 1.94 kcal/mol for hamster and human P16, respectively). Since the value for most proteins falls within 5–15 kcal/mol [24], both proteins can be regarded as thermodynamically unstable.

A structural model of hamster p16 protein

In order to evaluate the effect of potential hamster P16 mutations on structure/function, a structural model of hamster P16 protein was constructed using human P16 as template [20,28]. Hamster P16 protein is composed of four ankyrin repeats, each of which exhibits a helix-turn-helix conformation (Fig. 7A). These four helix-turn-helix motifs are arranged into a helical bundle, while the loops linking the neighboring motifs are relatively flexible, but retain an orientation almost perpendicular to the helical axes [20]. Both the amino- and carboxyl-termini are very flexible and have been shown in other studies to contribute little to the core structure

Fig. 7. Structural model of hamster P16 protein. SWISS-MODEL software was used in this modeling and the template structure was the solution structure of human P16 protein (PDB code: 1A5E). (A) Model showing the secondary structure in which the helical regions are represented as red boxes. Each ankyrin repeat has two α -helices except for the first helix in the second ankyrin repeat which is a nascent stretch and is not shown as a red box. (B) Solvent-accessible surface of hamster P16 protein. Positive and negative charges are indicated by blue and red, respectively. The solid arrow points to the proposed “pocket” for CDK4 binding. In both figures, the N- and C-termini are top right and bottom right, respectively.

of P16 [20]. The solvent-accessible surface of hamster P16 protein contains clusters of charged groups, which have been suggested to contribute to the interactions with CDK4 or CDK6 (Fig. 7B). In addition, there is a “pocket” located on the right side of the molecule, which could be the CDK4 binding site. This pocket is composed of a series of conserved charged residues, including Glu 80 (on the left edge of the pocket), Asp 76, Gln 42 (to form the back wall), and Arg 38 (to form a right inner wall devoid of any negatively charged groups). The pocket exhibits a conformation that provides a polar region dominated by negatively charged side chains to facilitate the electrostatic interactions between Asp 76 and the Arg residue in the active site of CDK4 (Arg 24) or CDK6 (Arg 31), as revealed in the crystal structures of P16/CDK6 and P19/CDK6 complexes [29,30]. It has been shown that any change in this conserved residue, Asp 84 in human P16 (Asp 76 in hamster P16), completely disrupts the inhibitory activity of P16 [20,27].

On the basis of the above comparative studies between human and hamster P16 proteins, we conclude that hamster P16 behaves in a biologically indistinguishable fashion from human P16. Consequently, the Syrian Golden hamster appears to be an appropriate model for further evaluation of *p16* in relation to pancreatic tumorigenesis and therapy.

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