Defective Folding of Mutant p16\textsuperscript{INK4} Proteins Encoded by Tumor-derived Alleles\textsuperscript{*}

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p16\textsuperscript{INK4} is a specific cyclin D-dependent kinase inhibitor and a multiple tumor suppressor. Inactivation of p16 is frequent in both primary tumors and tumor-derived cell lines. We describe here the conformational properties and oligomerization state of seven mutant p16 proteins; all of them are deficient in function. Four of the seven proteins show significantly disrupted secondary structure and backbone folding. The other three adopt partially folded, molten globule-like conformations. These proteins have near-native levels of secondary structure, but lack the ability to undergo a cooperative thermal transition and are substantially less resistant to proteolysis than is wild type p16. At low concentrations, two of the seven proteins are monomers, three exhibit an apparent molecular weight between the value of a monomer and a dimer, and the other two aggregate significantly. Our results strongly suggest that defective protein folding and/or aggregation is a common mechanism for inactivation of p16.

Recent evidence suggests that protein folding defects are likely the molecular basis of an increasing number of human diseases (for examples, see Refs. 1–5; for reviews, see Refs. 6 and 7). Mutations in genes that alter the amino acid sequence of proteins may result in molecules that are unable to fold, fold into alternative conformations, or undergo uncontrolled aggregation. In this study, we focus on a small cyclin D-dependent kinase inhibitor known as p16\textsuperscript{INK4} (abbreviated as p16\textsuperscript{1}). p16 was originally identified as a factor that specifically binds to Cdk4, cyclin-dependent kinase 4 (Cdk4), was originally identified as a factor that specifically binds to Cdk4, and inhibits the activity of cyclin-dependent kinase 4 (Cdk4), preventing progression through the G1 phase of the cell cycle (8). Approximately 2 years ago, p16 was proposed to be a multiple tumor suppressor (9, 10). Since then, p16 inactivation has been found in numerous types of malignant tumors, with mechanisms including homozygous deletion, genomic rearrangement, missense mutation, and transcriptional silencing (for reviews, see Refs. 11 and 12). Among all lines of evidence indicating that p16 plays a crucial role in tumorigenesis, perhaps the strongest one is that germ line mutations of p16 were found in patients with familial melanoma and have been shown to co-segregate with predisposition to this disease (13). In addition, p16 knock-out mice have been generated which develop cancer early in life (14). A number of studies indicate that some tumor-derived p16 mutations lead to proteins that are essentially intact, but are nevertheless deficient in function (15–19). In order to understand the underlying molecular mechanism of these loss-of-function mutations, we decided to examine the conformational properties and oligomerization state of several mutant p16 proteins.

**EXPERIMENTAL PROCEDURES**

**Protein Production and Purification**—The wild type p16 cDNA clone was provided by Dr. David Beach (Howard Hughes Medical Institute at Cold Spring Harbor Laboratory). The gene was amplified by polymerase chain reaction, cloned into a T7-polymerase based expression vector, pAED4 (20, 21), and the DNA sequence was confirmed by dideoxynucleotide sequencing. Throughout this study, we used an N-terminal truncated version of p16, with its first 8 residues deleted (8, 16). This variant has been shown to be fully active in terms of Cdk4 binding and inhibition (19) and is more soluble than the full-length protein (22). Site-directed mutagenesis was carried out using the method of Kunkel et al. (23) and verified by DNA sequencing. Recombinant p16 and its mutant proteins were expressed in Escherichia coli strain BL21 (Novagen), by growing cells to an A\textsubscript{600} = 0.6–1.0 and inducing with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested 2–3 h after induction.

For protein purification, cells (typically from 2 liters of culture) were disrupted by sonication in 40–50 ml of buffer containing 50 mM Tris (pH 8.0), 25% sucrose, and 1 mM EDTA, followed by DNase treatment (5 μg/ml) and centrifugation (27,000 × g, 15 min). The pellet was washed twice, once with the same buffer used for cell disruption, and once with buffer containing 20 mM Tris (pH 8.0), 1% Triton X-100, and 1 mM EDTA. The resulting pellet (inclusion bodies) contained mostly p16. p16 protein remaining in the supernatant was recovered by precipitation after adjusting the pH of the supernatant to pH 6, followed by centrifugation. The two pellets were combined, resuspended in 8 M urea, 50 mM Tris (pH 8.5), 5 mM EDTA, and 50 mM DTT, and loaded onto a fast flow DEAE-Sepharose column equilibrated with 20 mM Tris (pH 8.5), 4 M urea, 1 mM EDTA, and 5 mM DTT. The column was eluted with a linear gradient of salt (0–0.5 M NaCl) in the same buffer containing 4 M urea. The fractions that contained p16 were combined and dialyzed extensively against 5% acetic acid.

Both the wild type and mutant p16 proteins were further purified by reverse-phase HPLC on a Vydac C18 preparative column using a shallow water-acetonitrile gradient (0.1% increase of acetonitrile per minute) in the presence of 0.1% trifluoroacetic acid. The inclusion bodies under denaturing conditions has been shown previously to refold spontaneously and yield biologically active proteins (22, 24). Purified p16s were stored in a desiccator at −20 °C. Only freshly dissolved proteins were used for all studies reported in this paper.

**Circular Dichroism (CD) Spectroscopy**—All CD experiments were carried out on a Jasco J-715 spectropolarimeter equipped with a thermoelectric temperature controller in buffer containing 10 mM Tris (pH 8.5), 1 mM EDTA, and 1 mM DTT. The far-UV CD spectra were recorded using a 1-mm path length cuvette for protein concentrations between 10 and 80 μM or using a 1-cm path length cuvette for protein concentrations below 10 μM. The 1-cm cuvette was also used to record temperature scans with 3–5 μM protein and a heating rate of 20–25 °C/min. In order to test for thermal reversibility, all temperature scans were repeated twice using the same sample. Similar features had to be observed in both scans. We routinely checked the sample purity after CD experiments by reverse phase HPLC. No chemical modification or degradation was found as a result of the measurement.

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of proteins were determined spectroscopically by absorbance at 280 nm in 6 M guanidine hydrochloride using a molar extinction coefficient of 1.394 × 10^4 for wild type and mutant p16s (except G101W) and an extinction coefficient of 1.963 × 10^4 for G101W (25).

Sedimentation Equilibrium—Sedimentation equilibrium experiments were carried out at 4 °C in a Beckman XL-A analytical ultracentrifuge with six-sector cells using two rotor speeds (20 and 28 k rpm). The protein solution was dialyzed against the running buffer before loading into sample compartments. The wild type p16 was spun at three protein concentrations under three different buffer conditions (Table I), whereas the mutant proteins were only spun at one concentration (−20 μM) under conditions used for the CD and proteolysis experiments. After 16–18 h of equilibration, the sample compartments were scanned at three different wavelengths (235, 260, 280 nm). The data were combined and analyzed by a nonlinear least square fitting program H1D4000 (26) using values of partial specific volume calculated according to the protein's amino acid composition (27).

Limited Proteolysis—Limited proteolysis was carried out on ice in order to maximize the stability of mutant proteins. The starting solution consisted of 10 mM Tris (pH 8.5), 1 mM EDTA, 1 mM DTT, and 30 μM protein. To this solution a 1:100 ratio (v/v) of proteinase K (1 mg/ml) was added. After a specific time period, 40-μl aliquots of the reaction mixture were removed, added to tubes containing 2 μl of 0.1 M PMSF, and frozen on dry ice. These samples were kept frozen until analysis by Tricine-SDS gel electrophoresis (28).

RESULTS

We have studied seven mutant p16 proteins. Six of these mutations are associated with melanoma (three are germ line mutations found in familial melanoma, two were isolated from primary melanoma tissues, and one was isolated from melanoma cell lines). The other (D74N) is a mutation that has been identified in esophageal and bladder cancers.

In order to assess the secondary structure and backbone folding of various mutant proteins, we carried out CD studies. Our conditions for the CD experiments were determined by two criteria: (1) under these conditions, wild type p16 must be a monomer in solution; and (2) it must exhibit a reversible thermal unfolding transition. These criteria (especially the second one) limited us to use of a slightly basic pH with no additional salt.

Fig. 1 shows the far-UV CD spectra of these mutant p16 proteins. Four of the seven proteins (D74N, R87P, H98P, and V126D) show significantly disrupted secondary structure as indicated by a pronounced CD minimum near 200 nm and a loss of signal at 222 nm compared to the value of the wild type protein (Fig. 1A). The other three mutant proteins (P81L, G101W, and P114L) have levels of α-helical secondary structure similar to that of wild type p16 (Fig. 1B). Our results on G101W and P114L are in general agreement with the results reported earlier (22). The small differences can likely be accounted for by different experimental conditions used in the respective studies.

It has been suggested that p16 molecules have a tendency to associate with themselves both in vitro and in vivo and that point mutations may significantly accelerate the rate of aggregation (22). In order to determine the oligomerization state of wild type and mutant p16 proteins, we performed sedimentation equilibrium studies. Our results show that the wild type p16 is a monomer under a reasonably broad range of conditions. However, only two of the mutant proteins are strictly monomers under the conditions used in our CD experiments (Table I). Among the rest, three mutant proteins show an apparent molecular weight greater than the value of a monomer but less than the value of a dimer, indicating that these proteins may undergo a monomer-dimer equilibrium. The other two mutant proteins form large aggregates.

Since several mutant p16 proteins are prone to aggregation, it is important for us to know whether features observed in the CD spectra reflect the intrinsic properties of the molecule or are caused by intermolecular association. We noted that between 3 and 30 μM, the CD spectra of mutant p16s are essentially independent of protein concentration (data not shown). This result suggests, although does not prove, that aggregation does not significantly change the shape of CD spectra.

For those mutant proteins having significant levels of secondary structure and perhaps a backbone conformation similar to that of wild type p16, we would like to determine whether these proteins have well-defined side chain packing. One way to monitor aromatic side chain interactions is to measure the near-UV CD spectra. Unfortunately, both tryptophan residues in p16 are likely exposed to solvent (22) and, consistent with this observation, the near-UV CD signal of p16 is weak and appears to be insensitive to mutations (data not shown).

As an alternative way to study the side chain conformation, we examined the temperature dependence of the CD signal at 222 nm. Native proteins with well-packed side chains usually undergo a cooperative thermal transition upon increase of temperature, whereas, for mutant proteins lacking significant secondary structure do not exhibit such cooperativity. As shown in Fig. 2, wild type p16 unfolds cooperatively at approximately 45 °C. In contrast, mutant P81L does not show a cooperative thermal unfolding, indicat-
Conformational Analysis of Mutant p16INK4A Proteins

Analyses were carried out as described under "Experimental Procedures." The samples were spun at two speeds, 20 and 28 k rpm, with buffer conditions and protein concentrations (at the time of loading) indicated in the table. Data were collected by computer and analyzed by nonlinear least square fitting. No systematic deviation was observed for wild type, D74N, and P81L. Deviations for R87P and V126D were relatively small and only occurred at the end of data sets.

| Protein   | Buffer conditions                   | Protein concentration | Apparent molecular mass |
|-----------|-------------------------------------|------------------------|--------------------------|
| Wild type | 10 mM phosphate (pH 7.0), 50 mM NaCl | 3, 10, 50              | 15.5 ± 0.6*              |
| Wild type | 10 mM Tris (pH 8.5), 1 mM EDTA       | 5, 15, 50              | 14.8 ± 0.8               |
| Wild type | 10 mM Tris (pH 8.5), 1 mM EDTA, 0.2 mM NaCl | 5, 15, 50              | 15.5 ± 0.6               |
| D74N      | 10 mM Tris (pH 8.5), 1 mM EDTA, 1 mM DTT | 20                    | 15.5 ± 1.2               |
| P81L      | 10 mM Tris (pH 8.5), 1 mM EDTA, 1 mM DTT | 20                    | 14.0 ± 1.0               |
| R87P      | 10 mM Tris (pH 8.5), 1 mM EDTA, 1 mM DTT | 20                    | 20.5 ± 1.2               |
| H98P      | 10 mM Tris (pH 8.5), 1 mM EDTA, 1 mM DTT | 20                    | 28.4 ± 2.1               |
| G101W     | 10 mM Tris (pH 8.5), 1 mM EDTA, 1 mM DTT | 20                    | >500 (pelleted)          |
| P114L     | 10 mM Tris (pH 8.5), 1 mM EDTA, 1 mM DTT | 20                    | 100                     |
| V126D     | 10 mM Tris (pH 8.5), 1 mM EDTA, 1 mM DTT | 20                    | 24.2 ± 1.7               |

* Variations in the table represent 95% confidence as estimated by the fitting program.

Fig. 2. Thermally induced unfolding of wild type (○) and mutant P81L (●), G101W (■), and P114L (▲). Experiments were carried out using samples containing 3–5 μM protein in the buffer used for the CD spectra with a heating rate of 20–25 °C per h. All temperature scans were reversible since cooling to the initial condition resulted in recovery of more than 90% of the starting signal. Similar unfolding curves were observed when the sample was subjected to a second scan.

DISCUSSION

On the basis of structural integrity, the mutant p16 proteins can be divided into two major classes. In the first class are D74N, H87P, H98P, and V126D. These proteins have disrupted secondary structure and backbone folding, which provide a straightforward explanation for their functional deficiency. The most interesting member of this class is D74N. This protein is a monomer in solution and carries a relatively conserved amino acid substitution. Asp-74 is located at the C-terminal end of the second ankyrin repeat, and, based on secondary structure prediction (30) and the NMR assignment of the wild type protein (22), Asp-74 is not involved in a secondary structure element. However, Asp-74 is crucial for the overall folding of the protein, because even a conservative change at this site can disrupt the secondary structure and the function of p16. This observation is supported by calculations of the amino acid covariance in the ankyrin repeat sequences, which suggest that the C-terminal end of an ankyrin repeat is important for structural stabilization.3

The other three members of this class all carry mutations that significantly change the physical and chemical properties of the original amino acid. Two of these mutant proteins (R87P and H98P) have prolines at positions that do not normally contain a proline; the third one (V124D) contains a hydrophobic to charged amino acid substitution. Since wild type p16 is not a very stable protein, with a free energy of unfolding only about 1.9 kcal/mol (22), it is not surprising that these mutations substantially disrupt the structure and folding of the molecule.

Of more interest is the conformational state of the second class of mutant proteins (P81L, G101W, and P114L). These proteins apparently have been blocked at an intermediate stage of folding, with near-native levels of secondary structure, but nevertheless are deficient in function (32). Since two of the three proteins in this class aggregate significantly, we will use P81L as a prototype for our discussion. The conformational

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properties of P81L closely resemble those of a molten globule, including high secondary structure content, loose side chain packing, an absence of cooperative thermal transition, and susceptibility to proteolysis. Point mutations that turn a native protein into a molten globule have been described previously (35, 36). Thus, similar mechanisms may be invoked to explain the loss-of-function phenotype observed in this class of mutant p16 proteins.

Compared to native proteins, molten globules have additional exposed hydrophobic surface areas, which often lead to aggregation (31, 37). Two of the three proteins in the second class aggregate even at low concentrations. This may contribute to the functional inactivation because formation of large aggregates will significantly reduce the effective concentration of protein monomers and the availability of Cdk4 binding sites. Perhaps the most striking result is that all mutant p16 proteins examined in this study are also structurally compromised. Our results demonstrated that these tumor-derived mutations globally destabilize the secondary structure and side chain packing in the entire p16 molecule rather than locally disrupting its Cdk4 binding site, indicating that protein misfolding and/or aggregation could be the underlying mechanism for some human malignant diseases.

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