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Analysis of $p16^{INK4a}$ and Its Interaction with CDK4

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The interaction between cyclin-dependent kinase 4 (CDK4) and its inhibitor $p16^{INK4a}$ (p16) was studied by random mutagenesis and yeast two-hybrid system. The gene encoding p16 was mutagenized randomly and the amino acid changes that affect the binding of p16 to CDK4 were identified. Several amino acid residues were shown to be important for the binding and many of these changes occur at residues conserved in all known human p16 family proteins Most of the mutant p16 proteins that failed to bind to CDK4 contained multiple amino acid changes, and these alterations were observed throughout the entire gene with no apparent mutational patterns or hot spots. Some of the mutations that moderately reduced the binding activity severely affected the kinase-inhibitory activity of p16.

The protein $p16^{INK4a}$ (p16) is an inhibitor of CDK4-cyclin D (1). It belongs to a family of four proteins (p15, p16, p18 and p19). They all contain four ankyrin repeats, bind to and inhibit CDK4 and CDK6 specifically (2,3). The p16 and p15 genes are mapped on chromosome 9p21; and p16 is implicated as the multiple tumor suppressor gene (MTSI) located in that region which is frequently altered in many forms of malignancies (2–4). Two differently spliced transcripts of p16 were identified and one of them is cell cycle regulated (5,6). The p15 transcript is upregulated by TGF-β treatment in certain cells (7).

Several lines of evidence support the theory that p16 functions in a feedback loop in regulating the retinoblastoma susceptibility gene product (Rb) (1) which is a critical regulator at the G1-S transition. Alterations of p16 and Rb have an inverse correlation in many cell lines (8) and high levels of p16 mRNA were observed in cells lacking Rb function (9). Studies have also shown that Rb negative cells are insensitive to the growth-suppressing activity of p16 (10).

Mutations of p16, including deletion, frame-shift, missense and nonsense mutations, were found in many tumor cell lines (4). However, changes in the p16 gene in primary tumors are significantly rarer except in several specific types of malignancies (2–3,11–16). These observations suggest that p16 is a tumor suppressor whose disruption plays a role in the tumorigenesis of several specific cancers. Some of the point mutations reported in the cancers have been shown to disrupt the normal function of the protein in vitro and in vivo (17–19).

In this study, we tried to identify the amino acid residues in p16 that are important for the interaction with CDK4. We mutagenized the p16 gene randomly and screened for p16 mutants that have either reduced or no interaction with CDK4 in a yeast two-hybrid system. The amino acid changes in these mutants were then determined by sequence analysis.

MATERIALS AND METHODS

Cells and media. Yeast strain HF7c (20) was used in the two-hybrid system. The yeast cells were maintained on YPD medium and yeast transformants were grown on SD (Trp-,Leu- and/or His-) media. Escherichia coli strain DH5α (BRL) and BL21 (Pharmacia) were used for plasmid propagation and the expression of GST-p16 fusion proteins, respectively.

Random mutagenesis of the p16 gene. The polymerase chain reaction (PCR) method was used to generate p16 genes containing random point mutations (21). The plasmid pGADLH-p16 (1) was used as the template; mutant p16 genes were

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cleaved by EcoRI/Sall, religated in pGADLH and transformed into E. coli. All the transformants were pooled and plasmids were prepared from them.

Screening for p16 mutants that have reduced interaction with CDK4 in the yeast two-hybrid assay. Plasmids pGBT8 and pGADLH were used for the expression of the Ga14-DNA-binding domain-CDK4 fusion ($Ga14^{bd}$-CDK4) and the Ga14-activation domain-p16 fusion (p16-$Gal4^{ad}$) in the yeast as described previously (1). The interaction was measured by the expression of the reporter gene, the β-galactosidase. After cotransforming the yeast with the pooled mutated p16-containing plasmids and the CDK4 containing plasmid, transformants that showed white or light blue (wild type p16 gave a blue color) were collected. Plasmids from these colonies were recovered and the sequence of the p16 gene in these plasmids were determined by Sanger's dideoxy method (United States Biochem. manual).

In vitro kinase assay. A reconstituted CDK4-cyclin D2 system was used in an in vitro kinase assay as described previously (1,22). Several p16 mutants are expressed as GST fusion proteins in E. coli using the vector pGEX-5X-3 (Pharmacia) and purified by glutathione affinity columns (Pharmacia manual). Different amounts of these fusion proteins were added to 20 ul of the kinase assay reaction containing 1 ul CDK4/cyclin D2 lysate, and the inhibitory effects were analyzed by SDS gel electrophoresis and autoradiography.

RESULTS

Effects of amino acid changes on the CDK4 binding activity of p16. The amino acid changes in p16 mutants and the affects of these changes on CDK4-binding are summarized in Table 1. The mutants that produced white colonies usually had very little CDK4-binding activity (less than 10% of the wild-type as measured by the β-galactosidase activities). Those that gave light blue colonies showed CDK4-binding activities ranging from 15% to 90% of the wild-type. Seven mutants contain single mutation; twelve have two mutations and the rest contain three or more mutations. Most of the mutants contain at least one mutation in the conserved ankyrin consensus sequence.

To deduce single amino acid changes that could reduce the binding of p16 to CDK4, we used the following two observations to exclude neutral changes: (1) amino acid changes at position 120 and after 135 do not affect the activity (19); (2) amino acid changes present in both a white and a blue clone are either neutral or contribute only partially to the reduced activity in the white clone. Through this kind of analysis, we have concluded that several amino acid changes can reduce the binding activity of p16 to CDK4 (Table 2, Fig. 1).

The important amino acid changes and the frequency of mutations in the p16 gene observed in these experiments are summarized in Fig 1. Point mutations can be seen throughout the entire p16 sequence. We did not observe any apparent pattern of mutations.

Reduced CDK4-binding activity severely affects p16's kinase-inhibitory activity in vitro. Some of the mutants can still bind to CDK4 but less efficiently. To test if these mutants can still inhibit

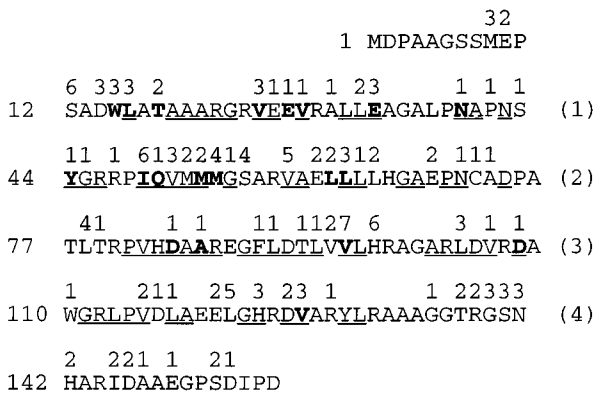

FIG. 1. Summary of potentially important mutations and the mutational frequencies observed in the experiment. The amino acid sequence of p16 is shown and the amino acid positions are indicated by the numbers on the left. The four ankyrin repeats are shown by the numbers on the right and the underlined residues are conserved (or with conserved substitutions) in all known proteins of the human p16 family. The residues whose change affects the activity (see table 2) are depicted as bold letters. Mutational frequencies are indicated above the affected amino acid residues.

TABLE 1
Effects of Amino Acid Changes on the Binding of $p16^{INK4a}$ to CDK4

Clone	Amino acid changes[a]	Color of colony[b]	Binding to CDK4[c]
P2	E26V, H98Y, D116G, L121P, N141D	w	3
p4	M9V, V59A, A86T, H98L, G139A, D146E	w	0.2
1-1	L63R	LB	55
1-2	I49N, F90Y	W	11
1-3	R47G, H98R, D125G, S140G, H142L, E149G	LB	13
1-4	S12P, V25A, M52V, E69K, H123R, I145L	LB	15
1-5	S12P, L16Q	LB	27
2-2	T18A, R138G	LB	11
2-3	V96E	LB	59
2-4	E10G, M54K	W	5
2-7	C72R, 1170	LB	26
2-8	L320, V126D	LB	22
3-1	I49T, V96A	W	6
3-2	V25A, L78P, L121P	W	5
4-1	E33K, N71D	LB	33
4-2	T18A, V59G, L63O, D84G	W	6
4-3	E33G, S56G, I145L	LB	32
4-4	D14G, V59A, H98L, N141I	LB	15
4-5	W15R	LB	43
4-6	S12T, L16P, S56C, V59A, V95A, L121N	LB	27
4-8	E33G	W	5
1	V25E, V51A, G135A	LB	39
2	L62P, L104P, D146G	LB	42
3	D14G, L94Q, A147P	LB	92
4	I49A, L104R, G139A	LB	80
5	L65P, L104Q	LB	49
6	Y44H	LB	44
7	S56C, L640, H98L, H123B	LB	77
8	P41S, M54V, L78P, V115E, S140G, S152P	LB	33
9	M9T, V51A, D153G	LB	64
10	D108G	LB	84
11	I49V, L91E, T93A, E120V	LB	79
12	E61G, V126A, I145K	LB	90
13	N39D, E120V, N141D	LB	48
14	L16R	LB	84
1′	D14G, Y44N, A73P	LB	NA
2′	M52T, V126D, T137A, R138G, G139S	W	NA
4′	M9T, L16P, I49T, M52K, T79A	LB	NA
5′	L62P, V96E, S140R	W	NA
6′	V59E, S56G, V95E	LB	NA
7′	E10G, S12P, G45C,	LB	NA
W1	E61G, L65P, E69G, L78P, L121N, T137A	w	NA
W3	E27G, M54V	W	NA
W4	W15R, S43G, M54K, V96A, H98L	W	NA
W8	M53T, L78P, D125V	W	NA
W9	V28E, H142R	W	NA
W10	W15R, I49T	W	NA
W11	S12T, L32P, E61V, L121P	W	NA
W12	M53K, H123P, R128I	W	NA
W13	A30V, M53T, G55D, L63P, S152T	W	NA
W14	Q50R, V115A	W	NA

[a] The amino acid changes in the ankyrin consensus sequence are underlined.
[b] W: white colonies; LB: light blue colonies (the wild type p16 produces a dark blue color).
[c] The binding is measured as the percentage of the wild type β-galactosidase activity. NA: not analyzed.

TABLE 2
Summary of Mutations That Affect Binding of p16 to CDK4

Mutations[a]	Clone	Clones used to exclude neutral changes[b]
L63R	1-1	—
V96E	2-3	—
W15R	4-5	—
E33G	4-8	—
Y44H	6	—
D108G	10	—
L16R	14	—
V28E	W9	H142R is neutral
N39D	13	E120V and N141D are neutral
T18A	2-2	R138G is neutral
V126D	2′	1-4, changes > 135 are neutral
L62P	5′	2-3, S140R is neutral
A86T	p4	7, 9, 4-4
I49T	3-1	2-3
M54K	2-4	7′
D84G	4-2	4-6, 2-2, 1-1
V25A	3-2	8, 4-6
M53T	W8	1-3, 8
O50R	w14	8
		8
E27G	W3	

[a] Mutations that reduce the binding of p16 to CDK4 are listed. The underlined changes occur at sites conserved in all human p16 family proteins and the bolded changes occur at sites with conserved substitutions in the p16 family.

[b] We used previous results that mutations at E120 and delition of residues after 135 are neutral (19) to exclude some of the neutral changes. We also used some of the blue clones that share mutations in a white clone to deduce that certain changes in the white clones can reduce the binding activity.

the kinase activity of CDK4, we chose several clones that showed binding activities ranging from 40 to 80% of the wild-type p16 and performed in vitro kinase assays. As shown in Fig.2, mutant clone #1 which retained about 40% binding activity had very little kinase-inhibitory activity at concentrations that the wild-type p16 would inhibit the kinase activity completely. Similar results were also observed on several other light blue clones that we examined (data not shown).

DISCUSSION

The p16 protein is a tumor-suppressor that plays an important role in G1 check point regulation through its CDK4/CDK6 inhibitory activity (2,3). Alterations of p16 have been reported in several

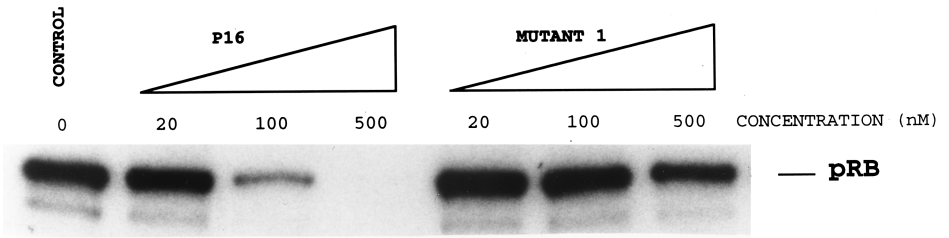

FIG. 2. In vitro assay of the CDK4 kinase activity. The in vitro kinase assay was done as described in the Materials and Methods section. This autoradiogram shows the phosphorylated pRB band. The concentrations of p16 or mutant p16 used in the reaction are indicated at the top.

cancers (11–16). In this study, we tried to identify the amino acid changes that affect the activity of the protein. Our results showed that several point mutations can either reduce or eliminate the activity of p16, and many of them occur at sites conserved in all human p16 family members (Table 2 and Fig. 1).

Due to the complexity of the mutations, importance of each amino acid change is difficult to discern except in those cases where either a single change is observed or some neutral changes can be excluded by other observations. Our previous study showed that the deletion of the c-terminal portion after codon 135 had no effect on the activity of p16 in vitro (19); therefore, we hypothesize that the amino acid changes observed in that region should have no effect on the activity. In most of the mutants, at least one change in the conserved ankyrin sequence occurred. These changes may be more important than others because we previously showed that point mutations in the conserved ankyrin sequence (observed in cancers) affected the activity while mutations in other regions had no apparent affect (19).

Analysis of mutational frequencies showed that several amino acid substitutions in the ankyrin repeats occurred more than six times while other mutations occurred 1–3 times (Fig. 1). However, we did not observe any apparent pattern or hot spot. Probably many amino acids throughout the four ankyrin repeats which includes 80% of the protein, are important for the activity of the protein.

Although many of the mutants (light blue colonies) still retained substantial CDK4-binding activity (Table 1), our in vitro kinase assays showed that they had much less inhibitory activity than the wild-type p16 (Fig. 2). These results are consistent with our previous observation that the G101W mutation observed in familial melanoma had much reduced kinase inhibitory activity despite its 74% binding activity (as compared to the wild type p16) (19). This mutant also had a reduced growth suppressing activity when expressed in mammalian cell lines (17). These observations suggest that changes that slightly affect the binding of p16 to CDK4 can still disrupt the normal function of p16.

In conclusion, we have examined fifty p16 mutants generated by random mutagenesis. These mutants have reduced or no CDK4-binding activity. We have identified several amino acids that are important for the activity of the protein. Although most of the mutants have multiple mutations that made it difficult to delineate the specific change that affected the activity, they usually contain at least one change in the conserved ankyrin sequence. For some of the mutants, we also showed that a moderate reduction in binding activity leads to a big reduction in the kinase-inhibitory activity.

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