Identification of the active region of the DNA synthesis inhibitory gene p21^{Sdi1}/CIP1/WAF1

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The cloning of the negative growth regulatory gene, p21^{Sdi1}, has led to the convergence of the fields of cellular senescence, cell cycle regulation and tumor suppression. This gene was first cloned as an inhibitor of DNA synthesis that was overexpressed in terminally non-dividing senescent human fibroblasts (*SDI1*) and later as a p53 transactivated gene (*WAF1*) and a Cdk-interacting protein (*CIP1*, p21) that inhibited cyclin-dependent kinase activity. To identify the active region(s) of p21^{Sdi1}, cDNA constructs encoding various deleted forms of the protein were analyzed. Amino acids 22–71 were found to be the minimal region required for DNA synthesis inhibition. Amino acids 49–71 were involved in binding to Cdk2, and constructs deleted in this region expressed proteins that were unable to inhibit Cdk2 kinase activity *in vitro*. The latter stretch of amino acids shared sequence similarity with amino acids 60–76 of the p27^{Kip1} protein, another Cdk inhibitor. Point mutations made in p21^{Sdi1} in this region confirmed that amino acids common to both proteins were involved in DNA synthesis inhibition. Additionally, a chimeric protein, in which amino acids 49–65 of p21^{Sdi1} were substituted with amino acids 60–76 of p27^{Kip1}, had almost the same DNA synthesis inhibitory activity as the wild-type protein. The results indicate that the region of sequence similarity between p21^{Sdi1} and p27^{Kip1} encodes an inhibitory motif characteristic of this family of Cdk inhibitors.

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and Nurse, 1990; Reed, 1992; Pagano *et al.*, 1993; Tsai *et al.*, 1993) and also for mitosis (Nurse, 1990). The growth inhibitory protein p21^{Sdi1} (Smith, 1992; Noda *et al.*, 1994) appears to act by inhibiting the kinase activity of various Cdks (Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993a). Immunoprecipitation with antibodies against different cyclins and Cdks has demonstrated that p21^{Sdi1} associates with several cyclin–Cdk complexes *in vivo* (Xiong *et al.*, 1992; M. Nakanishi *et al.*, submitted). These complexes are disrupted following cellular transformation with DNA tumor viruses (Xiong *et al.*, 1993b), suggesting a mechanism by which oncogenic proteins can alter the cell cycle of transformed cells. Wild-type p53 has been found to transactivate p21^{Sdi1} directly (El-Deiry *et al.*, 1993), indicating that it is a major downstream effector of this tumor suppressor gene. These results implicate p21^{Sdi1} not only as a negative regulator during normal cell proliferation but also as a potential tumor suppressor and, indeed, overexpression of p21^{Sdi1} can inhibit the proliferation of various tumor cells in culture (El-Deiry *et al.*, 1993).

Additional Cdk inhibitors, p16^{INK4A}, p15^{INK4B} and p27^{Kip1}, have also recently been cloned (Serrano *et al.*, 1993; Hannon and Beach, 1994; Polyak *et al.*, 1994a,b; Toyoshima and Hunter, 1994). p16^{INK4A} and p15^{INK4B} appear specifically to inhibit cyclin D–Cdk4 and cyclin D–Cdk6 complexes. In contrast, p27^{Kip1} inhibits a variety of cyclin–Cdk complexes in a manner similar to p21^{Sdi1}. Interestingly, p27^{Kip1} and p21^{Sdi1} share a region of similarity in their N-terminal sequences (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994) that is not present in p16^{INK4A} or p15^{INK4B}, indicating that there must be at least two mechanisms for Cdk inhibition.

To elucidate the mechanism of cyclin-dependent kinase inhibition of p21^{Sdi1} better we performed the studies described here to identify the active inhibitory region(s). The results implicate amino acids 42–71 as important for growth inhibition and suggest that the minimum amino acid sequence required for DNA synthesis inhibition spans amino acids 22–71, whereas amino acids 49–71 are involved in actual binding to Cdk2 and inhibition of kinase activity. Additionally, the data indicate that the region of similarity between p21^{Sdi1} and p27^{Kip1} involves a growth inhibitory motif that may define a family of Cdk inhibitors.

Results

Truncation of the C- and N-terminal regions of p21^{Sdi1} indicates that amino acids 22–71 are necessary for DNA synthesis inhibition

In order to determine the region(s) of the molecule that was required to inhibit DNA synthesis, we first constructed a series of cDNAs, expressed from the CMV promoter, which were progressively truncated from either the 3' or

Introduction

Control of cell proliferation in eukaryotes from yeast to humans involves the regulated synthesis, activation and degradation of a family of cyclins that act as the regulatory subunits of protein kinases termed cyclin-dependent kinases (Cdks) (Cross *et al.*, 1989). The Cdks are necessary for the initiation and progression through S phase (Blow

Fig. 1. C- and N-terminal truncation of p21^{Sdi1}. The indicated C- and N-terminal deleted mutants were transfected into normal human fibroblasts. Mutants were generated using restriction enzymes that have unique sites within the p21^{Sdi1} coding sequence (see Materials and methods). The amino acids encoded by the mutants are indicated at the N- and C-termini. Internal deletions are represented by black boxes. Cells were co-transfected with 1 μg pCMVβ-gal, 3 μg pBluescript as carrier DNA, and 1 μg of each mutant or 1 μg pCMV vector alone as a control. Percentage inhibition of DNA synthesis was reported as the average from two or more independent experiments.

5' end of the coding region (Figure 1). In the case of the 3' deletions, the truncated constructs encoded the N-terminal portion of the protein up to and including amino acids 122, 82, 71, 52 and 16, respectively. 5' deletions resulted in constructs expressing the C-terminal regions of the protein from amino acids 17, 22, 32 and 42, respectively. Each construct was tested for the ability to inhibit the initiation of DNA synthesis when transfected into HCA2, normal human fibroblast cells.

Transfection of the full-length coding region resulted in a ~90% decrease in the percentage of cells incorporating tritiated thymidine (Figure 1). Truncated polypeptides encoding the N-terminal 122, 82 and 71 amino acids were as effective as the full-length 164 amino acid protein in blocking entry into S phase, whereas a more severely truncated form, encoding only the first 52 amino acids, was completely inactive (Figure 1). This suggested that a potential critical region for the DNA synthesis inhibitory activity was located between amino acids 1 and 71. The role of the N-terminal portion of the protein in inhibiting entry into S phase was further clarified by deleting amino acids from the N-terminal region of the protein. Deletion of amino acids 1–16 had minimal effect on the inhibitory activity of the protein, whereas deletion up to amino acid 22 resulted in a drop in inhibition to approximately half that of the full-length protein. Deletion to amino acid 32 resulted in ablation of the DNA synthesis inhibitory activity of the molecule. These data suggested that amino acids 22–71 encompassed the minimum region of the protein required for DNA synthesis inhibitory activity.

This interpretation was confirmed by the fact that a construct encoding amino acids 22–71 was capable of minimal inhibition of DNA synthesis, and that a construct encoding amino acids 17–71 had about half the activity of the 17–164 amino acid polypeptide, which was as active as the complete protein. Additionally, a protein deleted in amino acids 17–52 had lost all inhibitory activity (Figure 1).

Internal deletions reveal that the region involved in Cdk2 binding and inhibition of kinase activity lies between amino acids 49 and 71

To map the DNA synthesis inhibitory region of the molecule more finely, small deletions were introduced in the N-terminus of the molecule. cDNAs encoding a series of p21^{Sdi1} proteins with six amino acid deletions (24–29, 30–35, 42–47, 53–58 and 66–71) were constructed. These constructs were fused in-frame with a portion of the hemagglutinin (HA) molecule to provide a unique antigen for immunostaining, which allowed us to determine that the protein products were expressed and where they localized in the cell. To ensure that the HA tag did not interfere with DNA synthesis inhibitory activity, the cDNAs encoding either the full-length 164 amino acid protein or amino acids 1–71 were also fused to the HA sequence at the C-terminus. The inhibitory activity of these cDNAs was similar to that of constructs without the HA tag (Figures 1 and 2A). Another control construct, deleted in amino acids 17–52, had no inhibitory activity whether it was HA tagged (Figure 2A) or not (Figure 1). Interestingly, cDNAs tagged with HA at the N-terminus exhibited a significant decrease in growth inhibitory activity (data not shown).

The various constructs were transfected into MDAH041 cells which do not express detectable levels of p21^{Sdi1}. They therefore provided a sensitive assay for small changes in DNA synthesis inhibitory activity that might be expected in the case of these minimally modified proteins. Deletion of amino acids 53–58 resulted in the greatest loss of DNA synthesis inhibitory activity, which was ~50% that of the full-length cDNA, when 1 μg DNA was transfected (Figure 2A). Another deletion, 42–47, also exhibited a slight decrease in activity. Since the possibility existed that a particular construct that was not inhibitory at physiological levels would cause inhibition when a large amount of DNA was transfected, we reduced the amount of plasmid DNA to 200 ng per transfection. It then became clear that a deletion in amino acids 42–47, 53–58 or 66–71 resulted in significant loss in ability to inhibit DNA synthesis (Figure 2A). Deletions 24–29 and 30–35 had activity nearly equivalent to that of wild-type at both DNA concentrations. These results suggest that the region of the protein critical for growth inhibition must lie between amino acids 36 and 71.

One possible explanation for the decreased inhibitory activity of a deleted gene is that resulting changes in tertiary structure of the protein product cause it to become less stable when expressed in cells. We therefore performed Western analysis with antibodies against the HA tag on extracts from cells co-transfected with the various deletion constructs and the β-galactosidase gene, using β-galactosidase activity to normalize protein loading. The results indicate that the differences in inhibitory activity

Fig. 2. Analysis of the N-terminal region of p21^{Sdi1}. A series of HA tagged six amino acid deletions were introduced into the 5' region of the p21^{Sdi1} cDNA by PCR (see Materials and methods). (A) MDAH041 cells were transfected by the method described in Figure 1 using 1 μ g (white bar) or 0.2 μ g (black bar) DNA of each of the indicated deletion mutants. When transfections were performed with 0.2 μ g, the total amount of DNA was adjusted to 5 μ g by the addition of 0.8 μ g pCMV vector (no insert). DNA synthesis inhibition was reported as the average of three independent experiments; standard deviations are indicated. (B) Western analysis of cells transfected with the indicated deletion mutant cDNAs. Extracts were loaded on the basis of equal amounts of β -galactosidase activity to normalize transfection efficiency. Proteins were detected with anti-HA tag antibodies as described in Materials and methods. (C) The various deletion mutants of p21^{Sdi1} were translated in rabbit reticulocyte lysates (Promega) and labeled with [³⁵S]methionine during translation. 5 μ l of each lysate was separated by SDS-PAGE (15% polyacrylamide), and ³⁵S-labeled proteins were detected by autoradiography. These lysates were used for the Cdk2 binding assay described in Figure 2D. (D) *In vitro* binding of the various deletion mutants of p21^{Sdi1} with Cdk2 was determined by adding 45 μ l of each reticulocyte lysate shown in Figure 2C to 1 μ g of Cdk2 purified from a baculovirus expression system (a kind gift from David O.Morgan) and immunoprecipitating with a polyclonal Cdk2 antibody. The immunoprecipitated proteins were separated by SDS-PAGE (12.5 % polyacrylamide), and ³⁵S-labeled proteins were detected by autoradiography (–, no Cdk2 added; +, Cdk2 added).

of the various deletion mutants cannot be attributed to the level of expression or stability of the protein (Figure 2B). In some cases deleted constructs were capable of DNA synthesis inhibition despite expression of lower amounts of protein (e.g. 24–29), and the reverse was also observed (e.g. 53–58).

To determine whether the DNA synthesis inhibition, or lack thereof, caused by these mutants occurred through interaction with Cdks, we examined the binding of *in vitro* translated mutant protein products with purified Cdk2 protein, since Cdk2 has been shown to be required for the initiation and progression of DNA synthesis (Pagano *et al.*, 1993; Tsai *et al.*, 1993). All mutant constructs produced intact protein of the expected molecular weight of 23 kDa (Figure 2C). Incubation of recombinant Cdk2 with *in vitro* translated p21^{Sdi1} mutant proteins, followed by immunoprecipitation with Cdk2 antisera, revealed that the wild-type protein and deletions 24–29, 30–35 and 72–77, co-precipitated with Cdk2, and 42–47 bound weakly to Cdk2 (Figure 2D). In contrast, the mutants deleted in 53–58 and 66–71, which had decreased inhibitory activity, did not bind Cdk2 (Figure 2D). To determine whether loss of Cdk2 binding correlated with Cdk2 kinase activity inhibition, we prepared GST fusion proteins of various deletion mutants for use in *in vitro* Cdk2 kinase inhibition

assays. In general, the results demonstrate a direct relationship between lack of Cdk2 binding and loss of kinase inhibition in the majority of the mutants studied, although there were some exceptions (Figure 3). For example, deletion of amino acids 30–35 and 72–77 resulted in proteins that appeared to bind more strongly to Cdk2 than wild-type Sdi1, and deletion of amino acids 42–47 resulted in weaker Cdk2 binding than the wild-type protein. However, all three of these mutants retained the ability to inhibit Cdk2 kinase activity to almost the same degree as wild-type Sdi1. These observations might be explained by the differences in the systems used to assay for binding activity and kinase inhibition. The binding assay consists of only two components, purified Cdk2 and the deletion mutant, but because the kinase inhibition assay involves the binding of the mutant proteins to intact cyclin–Cdk complexes, the relative affinities of the mutants for the cyclin–Cdk complex might be different from those for the Cdk alone.

Active inhibitory proteins localize to the nucleus, inactive proteins are in the cytoplasm

The intracellular localization of p21^{Sdi1} has been reported to be nuclear and there is a putative nuclear localization signal at the 3' end of the coding region of the gene (El-

Fig. 3. Cdk2 kinase inhibition activity of wild-type and deletion mutant recombinant GST-Sdi1 fusion proteins. Various amounts of GST fusion proteins, in a range from 10 ng to 10 μ g, were incubated with HeLa cell lysate and Cdk2 immunoprecipitates assayed for histone H1 kinase activity as detailed in Materials and methods.

Deiry *et al.*, 1993; Harper *et al.*, 1993; Noda *et al.*, 1994). However, a C-terminal truncated protein (containing only amino acids 1–71), which lacks this signal, had almost

the same inhibitory activity as the wild-type protein (Figure 1). We therefore determined the localization of the protein products of the various deletion mutants

Fig. 4. Intracellular localization of various deletion mutants of $p21^{Sdi1}$. One μ g of each deletion mutant plasmid was transfected into MDAH041 cells along with 4 μ g carrier DNA. Mutant proteins were detected by immunocytochemistry using a monoclonal antibody against the HA tag as described in Materials and methods.

following transfection of the cDNAs into MDAH041 cells. Deletion mutants with growth inhibitory activity localized primarily to the nucleus, whereas a high percentage of cells expressing inactive or less active proteins exhibited cytoplasmic staining (Figure 4). These data, along with the Cdk binding results, suggest that translocation of $p21^{Sdi1}$ into the nucleus may be dependent on the formation of a $p21$ -cyclin-Cdk complex. We have also determined that translocation of $p21^{Sdi1}$ to the nucleus does not appear to reflect the phase of the cell cycle, eliminating this as an alternative explanation for the lack of nuclear localization of some of the deletion mutants (M.Nakanishi *et al.*, submitted).

A region of shared sequence similarity between $p21$ and $p27$ is necessary for DNA synthesis inhibition

Recently, another Cdk inhibitor, $p27^{Kip1}$, was reported to have significant sequence similarity with the N-terminal region of $p21^{Sdi1}$, suggesting that these two proteins might share a common inhibitory mechanism. Since deletion analysis of $p21^{Sdi1}$ had indicated that loss of amino acids 42–71 resulted in decreased inhibition of DNA synthesis, and sequence comparison with $p27^{Kip1}$ had revealed regions of sequence similarity between amino acids 49 and 65 in $p21^{Sdi1}$ (Figure 5A) (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994), we constructed a further series of contiguous deletions spanning this region. Results

Table I. Point mutational analysis of the DNA synthesis inhibitory activity of $p21^{Sdi1}$

Mutation	Percent inhibition	Relative activity(%)
Wild-type	89.1 $\pm$ 3.2	100
E47G	79.4 $\pm$ 1.9	89.1
W49G	33.4 $\pm$ 9	37.5
F51G	44.5 $\pm$ 7.7	49.9
F53G	66.2 $\pm$ 6.4	74.3
T55A	86.3 $\pm$ 1.7	96.9
E56G	80.7 $\pm$ 2.6	90.6
P58A	77.3 $\pm$ 4.1	86.8
E60G	67.0 $\pm$ 1.7	75.2
D62G	83.5 $\pm$ 3.4	93.7
W65G	56.5 $\pm$ 5.1	63.4

from transient transfection assays demonstrated that removal of amino acids 49–53 resulted in a loss of most of the inhibitory activity of the protein, and deletion of amino acids 53–58 and 58–61 also resulted in significant loss of activity (Figure 5B). The stability of the mutant proteins was confirmed by Western analysis, which demonstrated that all of the deleted proteins were expressed in the transfected MDAH041 cells (Figure 5C). In addition, all deletion mutants made between amino acids 49 and 71 (e.g. 49–53, 53–58, 58–61, 61–66 and 66–71) did not bind to Cdk2 in *in vitro* binding experiments (data not shown, except for mutants 53–58 and 66–71, shown in Figure 2D).

However, since the native structure of the protein could be adversely affected by the creation of small deletions, we also performed site-directed mutagenesis in the *SDI1* cDNA in the region of sequence similarity to $p27^{Kip1}$ (amino acids 49–65). The results demonstrate that all point mutations in amino acids identical to those found in $p27^{Kip1}$ resulted in loss of inhibitory activity when compared with the wild-type protein (Table I). The most significant changes were observed following mutation of residues 49, 51 and 65. Some loss of inhibitory activity was also caused by mutation of residues 53 and 60. Interestingly, all point mutations in amino acids not identical to those in $p27^{Kip1}$ resulted in no loss of inhibitory activity.

We also constructed a chimeric plasmid in which amino acids 49–65 of $p21^{Sdi1}$ were replaced with amino acids 60–76 of $p27^{Kip1}$ (Figure 6A). This plasmid, when transfected into MDAH041 cells, inhibited DNA synthesis as efficiently as the $p21^{Sdi1}$ wild-type protein (Figure 6B), although the *in vitro* Cdk2-binding activity was somewhat less than that of wild-type $p21^{Sdi1}$ (Figure 6C and D).

Discussion

$p21^{Sdi1}$ is a novel DNA synthesis inhibitor cloned from senescent human fibroblasts (Smith, 1992; Noda *et al.*, 1994) which inhibits the activity of various Cdks *in vitro* (Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993a). A necessary step toward understanding how this gene regulates DNA synthesis was to determine the region of the protein involved in mediating inhibition of DNA synthesis. We therefore undertook the deletion analysis described here. The results demonstrate that only the N-terminal region of $p21^{Sdi1}$ is required for inhibition of cell

Fig. 5. Regions of sequence similarity between p21^{Sdi1} and p27^{Kip1} are necessary for inhibitory activity and Cdk2 binding. (A) Regions of sequence similarity in p21^{Sdi1} and p27^{Kip1}. (B) A series of four or six amino acid deletions were made using PCR as described in Materials and methods. The mutants were transfected into MDAH041 cells using 1 µg (white bar) or 0.2 µg (black bar) of each DNA as described in Figure 2A. Percentage inhibition of DNA synthesis was reported as the average of three independent experiments; standard deviations are indicated. (C) Western analysis of extracts from cells transfected with the indicated mutant cDNAs, performed as described in Figure 2B.

proliferation, and fine mapping studies implicate amino acids 22–71 as primarily involved in the negative growth effects. Interestingly, another Cdk inhibitor, p27^{Kip1}, has sequence similarity to p21^{Sdi1} in this N-terminal region and is also capable of arresting cells in G₁ following overexpression (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994). In addition, a C-terminal truncated p27^{Kip1} protein (containing amino acids 1–91) also has inhibitory activity similar to that observed with a C-terminus truncated p21^{Sdi1}. These results raised the possibility that the regions of sequence similarity shared by these Cdk inhibitors might be important for their inhibitory activity and that the mechanism of growth inhibition could be similar. Two distinct domains of similarity exist involving amino acids 17–33 in p21^{Sdi1} (28–44 in p27^{Kip1}) and amino acids 49–68 in p21^{Sdi1} (60–79 in p27^{Kip1}). The results of our deletion analysis of p21^{Sdi1} indicate that both domains are necessary for maximal inhibitory activity (Figure 1), and that amino acids 49–71 appear to be involved in DNA synthesis inhibition via Cdk2 binding and kinase inhibition (Figures 3 and 5). Site-directed mutagenesis revealed that mutation of single amino acids in the region of identity with p27^{Kip1} resulted in decreased inhibitory activity, when compared with wild-type protein (Table I). The most significant effect resulted from mutation in residues 49, 51 and 65, though mutation of amino acids 53 and 60 also caused some decrease in activity. Interestingly, mutations in amino acids not common with p27^{Kip1} exhibited minimal loss in inhibitory activity (47, 55, 56, 62). A chimeric protein, in which amino acids 49–65 of p21^{Sdi1} were substituted with amino acids 60–76 of p27^{Kip1}, had inhibitory activity almost equivalent to wild-type p21^{Sdi1} (Figure 6B) and could also bind Cdk2, although not as well as p21^{Sdi1} (Figure 6D). The decreased binding of Cdk2 by the chimeric protein might be explained on the basis of varying binding affinities for the different Cdk2s and/or the presence of non-conserved amino acids in the binding domain of the protein. These data also

suggest that the sequence WNFDXXXXPLEGXXXXW-XXV, the amino acid motif common to p21^{Sdi1} and p27^{Kip1}, and possibly other Cdk inhibitors that are yet to be discovered, is the active inhibitory region of these proteins.

In contrast, p16^{INK4A} and p15^{INK4B}, specific inhibitors of cyclin D–Cdk4 and cyclin D–Cdk6 complexes, have no significant sequence similarity with p21^{Sdi1} or p27^{Kip1}, indicating that they are not closely related. Both p16^{INK4A} and p15^{INK4B} have four ankyrin repeats, recently identified as a protein–protein interaction motif (Michaely and Bennet, 1992), that could mediate the action of these proteins.

A heat-stable protein kinase inhibitor, which strongly inhibits cyclic AMP-dependent protein kinase activity (A-kinase inhibitor), has been found to act via a pseudosubstrate region at the N-terminus (Scott *et al.*, 1985). A synthetic 20 amino acid peptide to this region, which contains the consensus sequence of the phosphorylation site, is as inhibitory as the intact protein (Wen and Taylor, 1994). However, p21^{Sdi1} does not appear to inhibit cyclin-dependent kinases by this mechanism, since it lacks the pseudosubstrate region.

Interestingly, DNA synthesis inhibition did not always correlate with Cdk2 kinase inhibition. In this regard, a recent report describes the direct binding of p21^{Sdi1} with PCNA and inhibition of DNA replication in an *in vitro* system (Waga *et al.*, 1994). This suggests that p21^{Sdi1}-mediated inhibition of DNA synthesis is a combination of both PCNA inactivation and inhibition of cyclin-dependent kinase activity. For example, deletion of amino acids 66–71 resulted in a protein that had no Cdk2 kinase inhibitory activity (Figure 3), but was still capable of inhibition of DNA synthesis (Figure 2A), suggesting that PCNA binding and inactivation may not require this portion of the protein. Another mutant deleted in amino acids 42–47 had Cdk2 kinase inhibitory activity similar to that of wild-type (Figure 3), but did not inhibit DNA synthesis to the same extent (Figure 2A), suggesting that

Fig. 6. Analysis of a chimeric p21^{Sdi1}/p27^{Kip1} protein. **(A)** Schematic of the p21^{Sdi1}/p27^{Kip1} chimeric construct. **(B)** The DNA synthesis inhibitory activity of wild-type p21^{Sdi1} and the chimeric construct of p21^{Sdi1}/p27^{Kip1} was determined by transfecting 1 µg (white bar) or 0.2 µg (black bar) of each plasmid into MDAH 041 cells as described in Figure 2A. **(C)** SDS-PAGE of *in vitro* translated p21^{Sdi1} and p21^{Sdi1}/p27^{Kip1} chimeric proteins from reticulocyte lysates performed as described in Figure 2C. Lanes 1 and 2 for each protein are 5 µl aliquots from duplicate lysates used in the Cdk2 binding assays in Figure 6D. **(D)** The binding of wild-type p21^{Sdi1} and the p21^{Sdi1}/p27^{Kip1} chimeric proteins to Cdk2 was demonstrated by incubating 45 µl of each *in vitro* translation reaction shown in Figure 6C with (+) or without (-) 1 µg of Cdk2 followed by immunoprecipitation with Cdk2 antiserum. The binding assay was performed as described in Figure 2D. *In vitro* translated proteins shown in Figure 6C, lanes 1 and 2, were used for Cdk2 - and Cdk2 + lanes, respectively.

these amino acids are involved in the direct binding and inactivation of PCNA. This possibility is supported by the observations that there is no sequence similarity to p27^{Kip1} in this stretch of amino acids and that p27^{Kip1} does not bind PCNA (Flores-Rozas *et al.*, 1994). Alternatively, it could be involved in some other function that contributes to DNA synthesis inhibition.

p21^{Sdi1} has been reported to localize in the nucleus (El-Deiry *et al.*, 1994). Extensive studies on the nuclear import of many viral and cellular proteins have revealed the existence of comparatively short sequences that function as nuclear localization signals (NLSs). These are generally identified by the fact that they are both necessary and sufficient for nuclear transport of a protein (Dingwall and Laskey, 1991; Garcia-Bustos *et al.*, 1991; Silver, 1991). The sequence of p21^{Sdi1} also has a putative NLS in the C-terminal region of the protein. However, a C-terminal truncated mutant, encoding only amino acids 1-71 and lacking the NLS, can still inhibit DNA synthesis and localize in the nucleus (Figures 1 and 4). These results suggest that the C-terminal NLS is not necessary for translocation of p21^{Sdi1} into the nucleus and that some other mechanism, possibly complex formation, is involved. This is supported by the immunocytochemistry results which demonstrated that deletion mutants capable of DNA synthesis inhibition localized to the nucleus of the cell, and that inactive forms of the protein tended to remain in the cytoplasm. Similar results have been observed in the case of cyclin A protein which normally accumulates in the nucleus immediately after synthesis (Girard *et al.*, 1991; Pines and Hunter, 1991; Pagano *et al.*, 1992; Zindy *et al.*, 1992). Deletion analysis has revealed a strong correlation between the regions of cyclin A required for transport to the nucleus and those involved in forming complexes with Cdk catalytic subunits (Maridor *et al.*, 1993), suggesting the possibility that nuclear localization of cyclin A requires complex formation with Cdk subunits. Alternatively, the interaction of p21^{Sdi1} with other molecules may contribute to its cellular localization and DNA synthesis inhibitory activity. For example, we have found that p21^{Sdi1} inhibits E2F-mediated transcription, even in cells that lack functional Rb (Dimiri *et al.*, manuscript in preparation), suggesting that p21^{Sdi1} interacts with one or more components of the E2F transcription complex.

In summary, our analysis has revealed an inhibitory motif common to two, and possibly additional, Cdk inhibitors. It also indicates that DNA synthesis inhibition by p21^{Sdi1} can occur by multiple mechanisms that include inhibition of Cdk activity, inhibition of DNA replication by binding to PCNA, breakdown of the E2F complex and possibly other mechanisms that remain to be determined.

Materials and methods

Cells and cell culture

HCA2, normal human diploid fibroblasts, were derived from neonatal foreskin in our laboratory. These cells achieve 80 population doublings (PD) before entering senescence and were used at PD 20-30 in all experiments. MDAH041 immortal skin fibroblast cells were derived from a patient with Li Fraumeni Syndrome. These cells do not express an active p53 gene product as a frame shift mutation causes premature termination in the N-terminal region of the molecule (Yin *et al.*, 1992). Details of cell culture conditions and estimation of population doublings have been described previously (Pereira-Smith and Smith, 1982).

Construction of deletion mutants of SDI1

pCMVsd1(1-686) was constructed by digesting the plasmid pCMVβ (MacGregor and Caskey, 1989) with *NotI* to remove the *Escherichia coli* β-galactosidase gene. This site was blunted with Klenow and an *SpeI* linker inserted to create pCMVSpeI. The full-length *SDI1* cDNA

was digested with *Bam*HI and *Dra*I and cloned into pBluescript. An *Spe*I linker was ligated into the *Hinc*II site of this vector to create *Spe*I ends for nucleotides 1–686 of the *SDII* cDNA. The *Spe*I fragment of *SDII* was then ligated into the pCMV*Spe*I vector to create pCMVsdil(1–686), containing the full 164 amino acid coding region for the wild-type *SDII* cDNA sequence. A series of mutants with nucleotide sequences encoding versions of p21^{SDII} truncated from the C-terminus were generated using restriction enzymes that have unique sites within the coding sequence. pCMVsdil(1–319) was generated by digesting pCMVsdil(1–686) with *Apa*I trimming the ends with T4 polymerase, followed by the insertion of a *Spe*I linker containing a stop codon in all three frames (New England Biolabs), (5'-CTAGACTAGTCTAG). The resulting plasmid was digested with *Spe*I and the liberated fragment encoding nucleotides 1–319 of the cDNA was inserted in to the *Spe*I site of pCMV*Spe*I. This expression plasmid encodes the first 82 amino acids of p21^{SDII} followed by ala-arg-leu-val. pCMVsdil(1–444) contains the first 122 residues of p21^{SDII} followed by leu-asp. pCMVsdil(1–127) used *Stu*I digestion and encodes a protein with the first 16 residues followed by ala-arg-leu-val. pCMVsdil(1–236) was derived from pCMVsdil(1–319) by digestion with *Tth*III, followed by filling in of the *Tth*III site and insertion of the *Spe*I linker, and encodes the first 52 amino acids of p21^{SDII}.

A series of mutants with nucleotide sequences encoding versions of p21^{SDII} truncated from the N-terminus were generated by standard PCR techniques (Higuchi, 1990). The following primer sets were used to introduce deletions:

- 1–16, GGAGGCGCCATGGCCTECCGCCGCTCTTC and GCGGCGCAGGCCATGGCGCCTCCTCTGAG;
 1–21, GGAGGCGCCATGTTTCGCCCCAGTGGACAGC and CACTGGGCCGAACATGGCGCCTCCTCTGAG;
 1–31, GGAGGCGCCATGCGCGACTGTGATGCGCTA and ATCAGATCGCGCATGGCGCCTCCTCTGAG;
 1–41, GGAGGCGCCATGATCCAGGAGGCCCGTGAG and GGCTCCTGGATCATGGCGCCTCCTCTGAG.

The internal deletion mutant pCMVsdil(128–235) was generated by successive *Stu*I and *Tth*III digestion of pCMVsdil(1–686), to delete nucleotides 128–235, followed by self-ligation of the circular vector. It encodes a protein containing the first 16 amino acids of p21^{SDII}, followed by residues 53–164.

For the deletion mapping studies in which only four to six amino acids were removed, a hemagglutinin (HA) tag was placed in-frame at the C-terminal of pCMVsdil(1–686). The HA tag sequence was introduced by PCR using the primers TTAGGCCTGTACGGAAGTG (splice site in pCMV vector) and TAGGAATTCACCTAGTCTAAGCGTAATCTGGAACATCGTATGGGTAGGGCTTCTCTTGGGA. Deletions were then introduced into the protein-coding region of this construct by standard PCR techniques (Higuchi, 1990). The HA tag allowed us to ascertain that the wild-type and mutated proteins were expressed in transfected cells and also to determine the cellular localization of the mutant proteins.

PCR primers were designed to delete four or six internal amino acids and to create a unique diagnostic *Xho*I or *Sma*I restriction site in the PCR product by adding the nucleotide sequence CCTCGAGGC or CCCCAGGGC in the deleted region. This resulted in a substitution of the amino acids Pro-Arg-Gly in place of the four or six naturally occurring amino acids. In one case (58–61) the nucleotide sequence TCCCGGGCC was used, resulting in the substitution of Ser-Arg-Ala because original sequence of amino acids 58–61 was Pro-Leu-Glu-Gly. The following primer sets were used to introduce deletions:

- 24–29, TTCGGCCCTCGAGGCCTGAGCCGCGACTGT and GCTCAGGCCTCGAGGCCGAAGAGGCGGCG;
 30–35, CGAGCAGCCTCGAGGCGCGCTAATGGCGGGC and TTAGCGCGCCTCGAGGCTGCTCGCTGTCCAC;
 42–47, GGCTGCCCTCGAGGCCGATGGAAGTTCGAC and CCATCGGCCTCGAGGGCAGCCGCCATTAG;
 49–53, CGTGAGCAGACCCCGGGGCGTACCGAGACACCACTG and CTCGGTGACGCCCCGGGTGCTCAGGGCCTCCTG;
 53–58, TTCGACCCTCGAGGCCTGGAGGGTGACTTC and CTCAGGCCTCGAGGGTGAAGTTCATCG;
 58–61, ACCGAGACATCCCGGGCCGACTTCGCTGGGAGCGT and GGCGAAGTCGGCCCCGGATGTCTCGGTGACAAAGTC

(substituted amino acids were Ser-Arg-Ala);

- 61–66, CCATGGAGCCCCGGGGCCGTGTGCGGGGCTTGGC and CCGCACACGGCCCCGGGGCTCCAGTGGTGTCTCGGT;
 66–71, GCCTGGCCTCGAGGCGGCCTGCCAAGCTC and CAGGCCGCTCGAGGCCAGGCGAAGTCACC;
 72–77, CGGGGCTTCCCCGGGGCCTTCCCACGGGGCCCCGGCGAGG and CGTGGGAAGGCCCCGGGGAAGGCCCGCACACGCTCCAG;

p21/p27 hybrid protein, CAGAATCACAAGCCACTCGAGGGTAAGTACGAGTGGGAGCGTGTGCGGGGCTT and CTTACCCTCGAGTGGCTTGTGATTCTGAAAGTCGAAGTCCATCGCTC. PCR primers were also designed to create point mutations. The following primer sets were used to introduce mutations: E47G, CAGGAGGCCCGTGGGCGATGGAAC and GTCCTCCGGGCACCCGCTACCTTG;

- W49G, GAGCGAGGGAACCTTCGACTTTGTGTC and CTCGCTCCCTTGAAGCTGAAACAG;
 F51G, GAGCGATGGAACGGCCAGTTTGTGTC and CTCGCTACCTTGGCGCTGAAACAG;
 F53G, TGGAACCTCGACCCCTGTACCCGAG and ACCTTGAAGCTGCCACAGTGGCTC;
 T55A, GACTTTGTGCGCCGAGACCACTG and CTGAAACAGCGGCTCTGTGGTGAC;
 E56G, GACTTTGTGACCCGGGACCACTG and CTGAAACAGTGGCCCTGTGGTGAC;
 P58A, GAGACAGCACTGGAGGGTACTTC and CTCTGTGCTGACCTCCCACTGAAG;
 E60G, GAGACACCACTGGGGGGTGACTTC and CTCTGTGGTGACCCCCCACTGAAG;
 D62G, CCACTGGAGGGTGGCTTCGCTCGG and GGTGACCTCCCAACGAAGCGGACC;
 W65G, GACTTCGCGGGGAGCGTGTGCGG and CTGAAGCGGCCCTCGCACACGCC.

All constructs were sequenced to verify that the desired deletions had been made, that the HA tag was intact and to confirm that no additional mutations had been introduced into the constructs. Following transfection, the deletion constructs were tested for protein production by immunocytochemistry using an anti-HA monoclonal antibody, 12CA5, obtained from Babco.

Transfection and determination of DNA synthesis inhibitory activity

HCA2 and MDAH 041 cells were co-transfected with 1 µg pCMVβ-gal and 1 or 0.2 µg of the plasmid DNAs described above using calcium phosphate precipitation (Chen and Okayama, 1987). pCMVβ-gal was used as a marker to detect transfected cells. Tritiated thymidine (1 µCi/ml) was added to the culture medium 24 h after transfection and the cells were incubated for an additional 36 h. The cells were fixed, stained for β-galactosidase activity, and processed for autoradiography to determine the percentage of β-galactosidase positive cells that had synthesized DNA (Noda *et al.*, 1994). Percentage inhibition was determined relative to control cells co-transfected with the pCMV vector and pCMVβ-gal.

Immunostaining

Cells were seeded on glass coverslips at $\sim 1 \times 10^4$ cells/cm² and allowed to adhere overnight. Following transfection, cells were incubated at 37°C for 24 h, washed twice with phosphate-buffered saline (PBS), and fixed in freshly prepared formaldehyde [4% (w/v) paraformaldehyde in PBS] for 15 min at room temperature. The fixed cells were then washed in PBS and incubated in 50 mM glycine in PBS for 10 min at room temperature. Cells were again washed with PBS and permeabilized by incubation in 0.2% Triton X-100 in PBS, at room temperature, for 10 min. After additional washings with PBS, immunostaining was performed using avidin-biotin-complex horseradish peroxidase (Vectastain Elite, Vector Laboratories). The primary antibody (12CA5) directed against the HA epitope was used at a 1:1000 dilution.

Western analysis and measurement of β-galactosidase activity

cDNAs encoding the various deletion mutants (5 µg) were co-transfected with pCMVβ-gal into MDAH041 cells in 25 cm² flasks using the calcium phosphate precipitation method described above. After 24 h

following transfection, the cells were washed twice in cold PBS and harvested in 250 µl reporter lysis buffer (Promega). In order to normalize the cell lysate for transfection efficiency, 50 µl of the extract was used for assay of β-galactosidase activity to calculate extract loading volumes (based on an equivalent amount of β-galactosidase activity) for SDS-PAGE (16.5% acrylamide). Following transfer to Immobilon membranes (Millipore), proteins were detected by incubation with monoclonal antibody 12CA5 (1:1500) and enhanced chemiluminescence (ECL, Amersham) using Kodak XAR-5 film.

In vitro binding of Cdk2

The various deletion mutants of p21^{Sdi1} were translated in reticulocyte lysates (Promega) using pBluescript-based plasmids as transcription templates for the T7 RNA polymerase. Labeling of translation products was performed using 40 µCi [³⁵S]methionine (1100 Ci/mM) per 50 µl reaction. For *in vitro* binding, 45 µl of the translation product was added to 500 µl binding buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 2 mM EDTA, 0.1% NP-40, 1 mM NaF, 0.1 mM sodium vanadate, 5 µg/ml leupeptin, 5 µg/ml soybean trypsin inhibitor, 5 µg/ml aprotinin) containing 1 µg Cdk2 protein purified from a baculovirus expression system. The mixture was gently rocked for 1 h at 4°C, and then 7.5 µg anti-Cdk2 antibody (rabbit polyclonal, Upstate Biotechnologies, Inc.) was added and mixing continued for 1 h at 4°C. The immunocomplex was absorbed by incubation for 1 h with 40 µl protein G plus A beads (Oncogene Science). The beads were then washed three times with 0.5 ml binding buffer prior to electrophoresis and autoradiography to detect the ³⁵S-labeled p21^{Sdi1}.

Cdk2 kinase inhibition assay

HeLa cell extract for Cdk2 histone H1 kinase assays was prepared by lysing cells in ice-cold lysis buffer containing 0.1% Nonidet P-40, 20 mM Tris-HCl (pH 7.40), 0.25 M NaCl, 1 mM EDTA, and the following protease inhibitors: 20 µg/ml soybean trypsin inhibitor, 2 µg/ml aprotinin, 100 µg/ml phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, 100 µM sodium orthovanadate, 50 mM sodium fluoride and 5 mg/ml *p*-nitrophenyl phosphate. Total HeLa cell lysate weighing 100 µg was incubated with the indicated amounts of various GST fusion proteins for 30 min at 37°C, and then Cdk2 was immunoprecipitated with 5 mg of rabbit polyclonal Cdk2 antiserum (Upstate Biotechnology Inc.) and assayed for histone H1 kinase activity. Quantification of histone H1 phosphorylation was accomplished by scanning dried polyacrylamide gels with a Betascope 603 Blot Analyzer.

Construction of wild-type and mutant Sdi1 GST fusion proteins

For the production of wild-type GST-Sdi1, pGEX-2T (Pharmacia) was digested with *Bam*HI and *Eco*RI. The full-length *SDI1* cDNA cloned in the *Bam*HI site of pBluescript (Stratagene) was digested with *Acl*I at nucleotide 76, blunted with Klenow, digested with *Eco*RI, and then ligated into *Sma*I/*Eco*RI-digested pBluescript. This plasmid was then digested with *Bam*HI and *Eco*RI and the *SDI1* fragment was ligated into the *Bam*HI/*Eco*RI-digested pGEX-2T vector. This plasmid was digested with *Bam*HI, blunted with Klenow, and a ten nucleotide *Xho*I linker was inserted to create a bacterial expression vector containing the entire *SDI1* coding sequence fused in-frame with the glutathione S-transferase peptide sequence. Six amino acid deletions were introduced into the wild-type GST-Sdi1 construct by standard PCR techniques using the following primer sets: 5' and 3' pGEX-2T vector primers, GCAGGGC-TGGCAAGCCACGTT and GAGCTGCATGTGTCAGAGGTT; 24-29, 30-35, 42-47, 53-58, 66-71 and 72-77 primers were the same as described previously except that in all these deletions a unique diagnostic *Sma*I site was created in the deleted region by the addition of the nucleotide sequence CCCCAGGGGC.

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Note added in proof

Recently, we have determined that the PCNA binding region of p21^{Sdi1} lies between amino acids 141 and 155 (M.Nakanishi *et al.*, manuscript in preparation).