The Protein SET Regulates the Inhibitory Effect of p21\(^{Cip1}\) on Cyclin E-Cyclin-dependent Kinase 2 Activity

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The cyclin-dependent kinase (CDK) inhibitor p21\(^{Cip1}\) has a dual role in the regulation of the cell cycle; it is an activator of cyclin D1-CDK4 complexes and an inhibitor of cyclins E/A-CDK2 activity. By affinity chromatography with p21\(^{Cip1}\)-Sepharose 4B columns, we purified a 39-kDa protein, which was identified by microsequence analysis as the oncoprotein SET. Complexes containing SET and p21\(^{Cip1}\) were detected in vivo by immunoprecipitation of Namalwa cell extracts using specific anti-p21\(^{Cip1}\) antibodies. We found that SET bound directly to p21\(^{Cip1}\) in vitro by the carboxy-terminal region of p21\(^{Cip1}\). SET had no direct effect on cyclin E/A-CDK2 activity, although it reversed the inhibition of cyclin E-CDK2, but not of cyclin A-CDK2, induced by p21\(^{Cip1}\). This result is specific for p21\(^{Cip1}\), since SET neither bound to p27\(^{Kip1}\) nor reversed its inhibitory effect on cyclin E-CDK2 or cyclin A-CDK2. Thus, SET appears to be a modulator of p21\(^{Cip1}\) inhibitory function. These results suggest that SET can regulate G\(_1\)/S transition by modulating the activity of cyclin E-CDK2.

The extracellular factors that regulate mammalian cell proliferation generate intracellular signals that ultimately converge on a family of serine-threonine kinases called cyclin-dependent kinases (CDKs)\(^1\) (1–3). CDKs, composed of regulatory cyclin and catalytic CDK subunits, are activated in a periodic manner during the cell cycle. Thus, cyclin D-CDK4 complexes are activated at mid G\(_1\), cyclin E-CDK2 complexes are necessary for G\(_1\)/S transition, cyclin A-CDK2 complexes are necessary for progression of DNA replication and cyclin B-CDK1 complexes are necessary for mitosis entry (4–6).

The activity of CDKs is regulated by the synthesis and degradation of cyclins at specific stages of the cycle, phosphorylation of specific amino acid residues of the CDK subunit, and binding of inhibitors (CKIs) that associate with cyclin-CDK complexes (1, 5, 7–9). Two families of CKIs have been described: INK4 and CIP/KIP (10). The INK4 proteins (p16\(^{INK4a}\), p15\(^{INK4b}\), p18\(^{INK4c}\), and p19\(^{INK4d}\)) bind specifically to CDK4 and to its homologue CDK6 (11–13). The CIP/KIP proteins (p21\(^{Cip1}\), p27\(^{Kip1}\), and p57\(^{Kip2}\)) bind to and inhibit the activity of a wide range of cyclin-CDK complexes (14–16). It is generally assumed that most of the biological activities of these CKIs depend on their ability to inhibit cyclin-CDKs. However, p21\(^{Cip1}\) also associates with and inhibits the proliferating cell nuclear antigen (PCNA), which could be important to block DNA synthesis following DNA damage (17).

The levels of p21\(^{Cip1}\) in quiescent cells are very low, but they are up-regulated in proliferating cells and also by antimitogenic stimuli (18, 19). p21\(^{Cip1}\) is also induced in some cell types during senescence and terminal differentiation (20). It is transcriptionally induced by the tumor suppressor protein p53 and plays an important role in cell cycle arrest induced by the activation of the G\(_1\) DNA damage checkpoint (21–23).

Recent evidence suggests that in addition to their role as cyclin-CDK inhibitors, p21\(^{Cip1}\) and p27\(^{Kip1}\) may also be activators of cyclin D-dependent kinases. p21\(^{Cip1}\) could promote the assembly of active cyclin D1-CDK4 complexes and provide a localization signal for their nuclear import (24). Primary mouse embryonic fibroblasts that lack genes encoding p21\(^{Cip1}\) and p27\(^{Kip1}\) fail to assemble detectable amounts of cyclin D-CDK complexes, express cyclin D proteins at much lower levels, and are unable to efficiently direct cyclin D proteins to the cell nucleus (25). Restoration of CKI function reverses all three defects and thereby restores cyclin D activity to normal physiological levels.

The dual role of p21\(^{Cip1}\) during the cell cycle suggests that its activity as inhibitor or activator must be highly regulated during the cell cycle and that other, still unknown, proteins might be involved in the modulation of p21\(^{Cip1}\) activity. Thus, we are searching for new p21\(^{Cip1}\)-binding proteins by using affinity chromatography with p21\(^{Cip1}\)-Sepharose 4B columns. We report here that the oncoprotein SET binds directly to p21\(^{Cip1}\) and that it reverses the inhibitory effect of p21\(^{Cip1}\) on cyclin E-CDK2 activity but not on cyclin A-CDK2.

EXPERIMENTAL PROCEDURES

Cell Cultures—Lymphoblastoid cell lines Molt-4 and Namalwa were obtained from the American Type Culture Collection. They were grown at 1 × 10\(^6\) cells/ml in RPMI 1640 (Flow Laboratories) supplemented with 10% fetal calf serum and 50 μg/ml of gentamicin as described previously (26).

Expression and Purification of Recombinant Proteins—The p21\(^{Cip1}\) cDNA was obtained by polymerase chain reaction from a human cDNA library, sequenced, and introduced into the pGEX-KG vector at NdeI–HindIII sites. SET cDNA was a generous gift from Dr. Damuni (New York). SET cDNA was introduced into the pGEX-KG vector at NcoI–HindIII sites. p27\(^{Kip1}\) cDNA was a generous gift from Dr. Massagué (New York). p27\(^{Kip1}\) cDNA was introduced into the pGEX-KG vector at NdeI–XhoI sites. To obtain recombinant glutathione S-transferase (GST), GST-p21\(^{Cip1}\) and GST-SET proteins, the BL21 LysE strain of E. coli was transformed with the vectors pGEX-KG, pGEX-KG-p21\(^{Cip1}\), and pGEX-KG-SET. A single colony was grown to saturation and activated by 0.5 mM isopropyl-β-D-thiogalactopyranoside for 16 h. To purify...
these fusion proteins, bacteria expressing these proteins were resuspended in NENT buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40). The samples were then sonicated for 10 s three times, centrifuged for 10 min at 27,000 × g, and finally resuspended in NENT buffer with or without Sarcosyl instead of Nonidet P-40. The lysates were incubated with glutathione-agarose beads. After three washes, the proteins were eluted with 20 mM reduced glutathione in 50 mM Tris-HCl, pH 9.0, and 120 mM NaCl.

**p21^Cip1/-Sepharose Affinity Chromatography**—The p21^Cip1/-Sepharose 4B columns were prepared by coupling 5–7 μg of purified GST-p21^Cip1^-GST protein to 3 ml of CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech). Control columns of purified GST were also constructed in the same way. Molt-4 cells were lysed in buffer A (50 mM Hepes-KOH, pH 7.6, 50 mM KCl, 1 mM MgCl₂, 1 mM dithiothreitol, and 1 μg/ml aprotinin). The extracts (10 mg) were then loaded on the GST-p21^Cip1-Sepharose or GST-Sepharose columns, and after washing in 50 vol of buffer A the bound proteins were eluted with the same buffer but containing 1 M KCl instead of 50 mM KCl.

**Gel Electrophoresis and Immunoblotting**—Samples were electrophoresed on SDS-polyacrylamide gels (SDS-PAGE) as described in Ref. 27. After electrophoresis, the proteins were transferred to Immobilon-P membranes for 2 h at 60 V. The membranes were preincubated in Tris-buffered saline (TBS) (20 mM Tris-HCl, pH 7.5, and 150 mM NaCl) containing 0.1% milk powder for 2 h at room temperature. The specific antigens were identified by using the following antibodies: anti-p21^Cip1 (C19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-cyclin A (C-22; Santa Cruz Biotechnology), anti-CDK2 (6-505; Upstate Biotechnology, Inc., Lake Placid, NY), anti-PCNA (1170406; Roche Molecular Biochemicals), and anti-SET (a generous gift of Dr. Copeland, National Cancer Institute) diluted in TBS containing 0.5% defatted milk powder and 1% bovine serum albumin. After washing in TBS, 0.05% Tween 20 and in TBS, the reaction was visualized by ECL (Amersham Pharmacia Biotech) or with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (promega). The band corresponding to the 39-kDa protein was then sliced from the gel and digested with trypsin. After digestion, the peptides were separated by high performance liquid chromatography, and a peptide was sequenced with an automated sequencer using protocols, reagents, chemicals, and materials from Applied Biosystems (Warrington, UK).

**Immunoprecipitation and Kinase Assays**—To determine whether the SET protein was associated with p21^Cip1, immunoprecipitation (IP) experiments were performed. Namalwa cells (1 × 10⁶ cells) were lysed in IP buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin) and incubated with 5 μg of a monoclonal anti-p21^Cip1 antibody (WAF-1 OP64, Calbiochem) overnight at 4°C. Protein immunocomplexes were then incubated with 10 μg of an alkaline phosphatase-(1:10,000 dilution; Promega) or a horseradish peroxidase-(1:2,000 dilution; Bio-Rad) coupled secondary antibody for 45 min. After washing in TBS, 0.05% Tween 20 and in TBS, the reaction was visualized by ECL (Amersham Pharmacia Biotech) or with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Promega). The unbound fraction was collected, whereas the bound proteins were eluted with 1 M KCl and subjected to SDS-PAGE. The eluates contained three major proteins of 39, 70, and 80 kDa and several minor proteins of a variety of sizes (Fig. 1A). These proteins were specifically bound to p21^Cip1 and not to GST, since control GST-Sepharose 4B columns did not retain them (Fig. 1A). Western blot analysis revealed that the eluates contained PCNA, cyclin A, and CDK2, indicating that the GST-p21^Cip1-Sepharose column worked properly, since it retained known p21^Cip1-binding proteins (Fig. 1B).

**Identification of the 39-kDa p21^Cip1-binding Protein as the Oncoprotein SET**—To identify the 39-kDa p21^Cip1-binding protein, we performed affinity chromatography analysis using GST-p21^Cip1-Sepharose 4B columns. For these experiments, we selected the lymphoblastoid cell line Molt-4, because it did not express p21^Cip1, and thus we avoid the association of the p21^Cip1-binding proteins of the cell extract with the endogenous p21^Cip1. The columns were washed with Molt-4 cell lysates and subsequently washed with loading buffer. The proteins associated with GST-p21^Cip1 were then eluted with 1 M KCl and subjected to SDS-PAGE. The 39-kDa protein was sliced from the gel and then microsequenced. A sequence of nine amino acids was obtained from this protein. This sequence matched perfectly (100% identity) with the amino acids 78–86 of the SET protein (A). Western blot analysis revealed that the eluates contained PCNA, cyclin A, and CDK2, indicating that the GST-p21^Cip1-Sepharose column worked properly, since it retained known p21^Cip1-binding proteins (Fig. 1B).
Role of SET on p21 Cip1 Activity—The direct interaction of SET with p21Cip1 suggested that SET could modulate the function of p21Cip1. Since p21Cip1 is a strong inhibitor of the activity of cyclin-CDK2 complexes, we analyzed whether SET can modulate the inhibitory effect of p21Cip1 on cyclin E-CDK2 or cyclin A-CDK2 activities. Thus, we immunoprecipitated Molt-4 cell extracts using specific anti-cyclin E or anti-cyclin A antibodies and measured CDK2 activity in the immunoprecipitates. In both cases, CDK2 activity was inhibited by the addition of 400 nM of recombinant GST-p21Cip1 (Fig. 5). We also observed that SET had no effect on cyclin E- or cyclin A-associated CDK2 activities at concentrations of 400 or 800 nM. However, when SET was added together with p21Cip1 it reversed the inhibitory effect of p21Cip1 on cyclin E-CDK2 activity but not on cyclin A-CDK2 (Fig. 5). This effect was specific, since purified GST did not reverse the p21Cip1-induced CDK2 inhibition.

We also analyzed the effect of SET on the inhibition of cyclin E-CDK2 or cyclin A-CDK2 by p27Kip1. Thus, IPs obtained using anti-cyclin E or anti-cyclin A antibodies were assayed for CDK2 activity using specific anti-SET or anti-GST antibodies.
More recently, it has been shown that calmodulin binds to cyclin D3, the p21Cip1 functions are performed by different domains of the protein. The NH2-terminal domain contains two regions, which blocks DNA synthesis (31). It has been also shown that p27Kip1 are necessary for certain processes that positively regulate cell cycle progression: cyclin D assembly and it is mainly located in the nucleus. Thus, it appears that p21Cip1 has a dual role in the regulation of cell cycle progression as activators of CDK4 and as inhibitors of CDK2 activity. To perform these different functions, the activity of p21Cip1 must be highly regulated during the cell cycle. One possible mechanism of regulation may be through additional components associated with cyclin-CDK-CKI complexes. Consistent with this idea is the observation of high molecular weight cyclin-CDK-CKI complexes in different cellular types (36). Although some of the proteins associated with these complexes are known, some others still remain unidentified (37, 38).

The CDK inhibitor p21Cip1 negatively regulates cell cycle progression and enforce a cell cycle arrest when overexpressed in the cells (15, 30). These effects are due to the binding of p21Cip1 to cyclin-CDK complexes, which leads to the inhibition of CDK activity and to the association of p21Cip1 with PCNA, which blocks DNA synthesis (31). It has been also shown that p21Cip1 and p27Kip1 are necessary for certain processes that positively regulate cell cycle progression: cyclin D assembly with CDK4, its stability, and its nuclear localization (24, 25). More recently, it has been shown that calmodulin binds to p21Cip1 in a Ca2+-dependent manner and that it regulates its nuclear localization but also that of cyclin D1 and CDK4 (32, 33). The p21Cip1 functions are performed by different domains of the protein. The NH2-terminal domain contains two regions, which specifically bind to cyclins and CDKs. The carboxy-terminal domain has a sequence that interacts with PCNA and which blocks DNA synthesis (31). It has been also shown that the terminal domain has a sequence that interacts with PCNA and which specifically bind to cyclins and CDKs. The carboxyl-terminal domain is essential for template-activating factor I activity (42). SET has been also identified as a potent inhibitor of the protein phosphatase P2A (43). Since P2A has been implicated in the regulation of cell cycle progression (44), it has been suggested that the SET-CAN gene fusion, which occurs in acute nonlymphocytic myeloid leukemia, may lead to altered regulation of P2A activity and thus contribute to leukemogenesis (45).

We report here that the oncoprotein SET associates with p21Cip1 in vivo and in vitro. Results also indicate that SET modulates the p21Cip1 inhibitory effect on cyclin E-CDK2 but not on cyclin A-CDK2 in vitro. SET was first identified as a gene that was fused to the CAN gene in a patient with acute undifferentiated leukemia, apparently as a result of a translocation (29). The SET-CAN fusion gene generates a transcript encoding a chimeric SET-CAN protein of 155 kDa. The SET protein shows similarity with the yeast nucleosome assembly protein NAP-I and is widely expressed in human and mouse tissues (29, 39, 40). It is phosphorylated in serine residues in vivo and it is mainly located in the nucleus.

Not much is known about the cellular role of SET, although several possible functions have been proposed. SET interacts specifically with B-type cyclins but not with cyclin A, although the functional significance of this interaction has not been elucidated (41). Moreover, SET has been found to be identical to template-activating factor I, a host protein necessary for DNA replication of the adenovirus genome (42). A large acidic domain in the C-terminal region of SET is essential for template-activating factor I activity (42). SET has also been identified as a potent inhibitor of the protein phosphatase P2A (43). Since P2A has been implicated in the regulation of cell cycle progression (44), it has been suggested that the SET-CAN gene fusion, which occurs in acute nonlymphocytic myeloid leukemia, may lead to altered regulation of P2A activity and thus contribute to leukemogenesis (45).

The known functions of SET clearly relate it with the control of cell cycle progression, although the steps regulated by SET remain unclear. Our results support the hypothesis that SET is a cell cycle regulator because of its ability to modulate p21Cip1 function, specifically on cyclin E-CDK2 activity. It is assumed that cyclin E-CDK2 activity is essential for the triggering of DNA replication (1). Thus, our results suggest that SET could be involved in the control of G1/S transition. Our results can be related with the reported effect of template-activating factor I (SET) on DNA replication in the adenovirus. The stimulatory effect of template-activating factor I on DNA synthesis could be...
due to the inactivation of p21Cip1 inhibitory function on cyclin E-CDK2 activity needed to trigger DNA synthesis. However, this possibility remains to be demonstrated.

The mechanism by which SET regulates cyclin E-CDK2 activity has not been elucidated. We showed that SET did not block the binding of p21Cip1 to cyclin E-CDK2 complexes. Moreover, we observed that SET was able to bind to cyclin E-CDK2 complexes in the absence of p21Cip1. This binding did not produce any alteration of cyclin E-CDK2 activity; thus, the SET-induced reversion of the inhibition of CDK2 activity by p21Cip1 complexes cannot be due to an increase in CDK2 activity induced by SET. The most likely explanation for the effect of SET is that its association with the cyclin E-CDK2-p21Cip1 complexes induces a conformational change in any of these proteins that overrules the inhibition of CDK2 activity by p21Cip1.

We found that the SET-binding domain of p21Cip1 is located at the carboxyl-terminal region. This fact is consistent with the evidence we report here showing that the binding of SET to p21Cip1 did not block the association of p21Cip1 with cyclin E-CDK2 complexes, since this association is mediated by p21Cip1 regions located at the NH2 terminus. Interestingly, the carboxyl-terminal domain contains a region, which binds PCNA and calmodulin (32, 35). Thus, although we still do not know the functional significance of this finding it suggest that SET could also modulate the action of p21Cip1 on PCNA and the role of calmodulin on p21Cip1-cyclin D-CDK4 complexes.

Although the functional relevance of the results reported here in the cell cycle progression in vivo are unknown, we can hypothesize that SET can regulate cell cycle progression at the G1/S transition. We can also speculate that the CAN-SET chimeric protein produced in acute nonlymphocytic myeloid leukemia may impair the normal regulation of p21Cip1 by wild type SET and that this process could also contribute to leukemogenesis.

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