A variant form of p27 was unexpectedly detected in a synchronized culture of NIH3T3 cells treated with serum. The expression levels of this form of p27 which lacked its amino (NH2-)terminal region reached maximum during G2/M phase. Since the appearance of the NH2-terminal truncated form of p27 coincided with increased expression of Cdc2, we hypothesized that p27 may play a role in regulating Cdc2 catalytic activity. To test this hypothesis, wild type p27, as well as the amino-terminal (Np27) and carboxyl-terminal (Cp27), were individually expressed, purified, and examined for their ability to regulate Cdk2 kinase activity in vitro. Our data showed that both p27 and Np27 inhibited Cdk2 kinase activity. However, in marked contrast, Cp27 enhanced the Cdk2 kinase activity. In vitro kinase assays showed that Cp27 and p27 were phosphorylated by Cdk2, whereas Np27 was not. In addition, we demonstrated that deletion of the putative Cdc2 phosphorylation site in the carboxyl-terminal domain of Cp27 diminished activation of Cdc2 kinase activity otherwise stimulated by Cp27. A similar deletion did not have any effect on the inhibitory function of p27. Together these results suggest that the carboxyl-terminal domain of p27 may activate Cdc2 kinase activity in vivo during G2/M and that this effect may be regulated by serine/threonine phosphorylation.

Molecular analysis of growth factor regulation of cell growth in mammalian cells has led to the identification of a new class of molecules that are involved in controlling cell cycle transition. Cyclin-dependent kinases (Cdks) are key regulators of cell cycle progression (1, 2), and their activities are positively regulated by their activating subunits, the cyclins. Cyclin molecules identified to date include cyclins A, B, C, D, E, F, G, and H. These molecules bind to Cdks with differential affinities and may play a role in regulating Cdc2 catalytic activity. To investigate the biochemical effect of different regions of p27 on activation of Cdc2 kinase activity, we hypothesized that p27 may contribute to their enzymatic activation. Accumulating evidence indicates that the G1/S transition is regulated by D-type cyclins (D1, D2, D3) and cyclin E activating the Cdk4/Cdk6 and Cdk2, respectively (3, 4), while cyclins A and B have been shown to play a role in the G2/M transition by activating Cdk1 (Cdc2) (5, 6). The activity of Cdk5 is also negatively regulated by a family of proteins collectively designated as cyclin-dependent kinase inhibitors (7–11). On the basis of structural features, these inhibitors can be divided into two subfamilies, namely the p16INK4a/p15INK4b/p18INK4c/p19INK4d and the p21Cip1/p27Kip1/p57Kip2. The main targets for p16 and related proteins are Cdk4 and Cdk6, and p16 family members act by inhibiting complex formation between these Cdns and D-type cyclins (12). In contrast, p21, p27, and p57 inhibit the function of multiple Cdk-cyclin complexes, including Cdk2, Cdk3, Cdk4, Cdk6, and Cdc2 without dissociating the Cdk-cyclin complex (13–15). Interestingly, the amino-terminal domains of p21, p27, and p57 have been shown to contain a conserved region that interacts with cyclin E-Cdk2, thereby inhibiting its activity (16). In contrast, the COOH-terminal domains of p21, p27, and p57 do not exhibit any structural or functional similarity. Indeed, the COOH-terminal domain of p21, unlike its counterparts in p27 and p57, has been shown to contain a binding site for proliferating cell nuclear antigen, a major factor required for DNA synthesis during G2/M cell cycle progression (17). In view of these observations, and lack of any data concerning the role of COOH-terminal region of p27 or p57, we sought to investigate the biochemical effect of different regions of p27 on activation of Cdc2 kinase activity. Our data provides evidence for a mechanism by which p27 may regulate Cdc2 kinase activity in vivo.

EXPERIMENTAL PROCEDURES

Human p27, Np27, Cp27, p27del, and p27del cDNAs were made by PCR using the following oligonucleotide pairs; I-II, I-IV, II-III, I-V, and III-V, respectively, listed as follows: I, ATGAGACAGAGATCCATGTCAGAGCAGAGTTGCGAC; II, ATGAGACAGAGTCGACCGTTTGACGATCATGTCAGAGTGGCGAC; III, ATGAGACAGAGGATCCGTGCCGGCGCAGGAGGGCA; IV, ATGAGACAGAGTCCGACCGCGCGCGACCTTGACCAGGC; V, ATGAGACAGAGTCGAGGTCTCCCAGAAGAGGCGTATTG. The PCR products were cloned into a bacterial expression vector (pQE9). Proteins were expressed in M15prep4 bacteria and purified under denaturing conditions as described earlier (18). Antibodies except p21 (OP76, Oncogene Science, Cambridge, MA) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) (cyclin A, sc-596; cyclin B, sc-245; cyclin D1, sc-753; cyclin D2, sc-593; cyclin D3, sc-182; cyclin E, sc-481; Cdc2, sc-54; Cdk2, sc-163; Cdk4, sc-260; Cdk5, sc-173; Cdk6, sc-177; p27, sc-928 and sc-527). Recombinant human Cdc2p34-cyclin B was obtained from New England Biolabs Inc. (Beverly, MA). Western blot analyses were performed as described previously (19). In vitro kinase assays were performed essentially as described previously (20) in 20-μl volume by using 1 unit of enzyme and 2.5 μM histone H1 (Boehringer Mannheim) at 30 °C for 30 min in the presence or absence of recombinant p27 constructs. Reactions were terminated by adding an equal volume of 2 × sample buffer. The reaction mixtures were electrophoretically separated by 12% SDS-PAGE. The gel was dried and autoradiographed. For enzyme kinetic studies, autoradiograms were scanned, and data were quantitated using QDS software.

RESULTS AND DISCUSSION

Regulation of Cell Cycle Machinery in Serum-stimulated NIH3T3 Cells—To better understand the molecular mechanisms of cell cycle progression in murine fibroblasts, quiescent NIH3T3 cells were treated with 10% fetal calf serum for varying periods of time, and the levels of key cell cycle regulators were analyzed by Western blot analysis. The results, summarized in Fig. 1, demonstrate that expression levels of Cdk2 and
Cdk-4 increased significantly around 4–6 h and remained high throughout the cell cycle. In parallel experiments cyclin E, cyclin D2, and cyclin D3 levels showed a similar increase, while the levels of Cdk5, Cdk6, and cyclin D1 stayed almost constant. Most notably the levels of cyclin A, cyclin B, and Cdc2 increased markedly at 12–14 h after serum stimulation. This coincided with increased cellular DNA content as detected by fluorescent-activated cell sorter analysis (data not shown). In parallel, we also investigated changes in the level of the Cdk inhibitors, p21 and p27 (Fig. 2). Western blot analysis showed an increase in p21 levels starting at 6–8 h, which remained high thereafter (Fig. 2A). In contrast, p27 levels showed a dramatic increase at 30 min poststimulation, followed by a gradual decrease to a minimal level at 12–14 h (Fig. 2, B and C). Surprisingly, the antibody generated to the COOH-terminal domain of p27 could detect not only the full-length p27, but also an additional protein of apparent molecular mass of around 22 kDa (Fig. 2C), which could not be detected by the antibody generated to the NH2-terminal domain of p27 (Fig. 2B). The steady state level of this form of p27 normalized for similar levels of full-length p27 reached its maximum levels at 22–24 h, around the same time the DNA content was the highest (data not shown). The specificity of the antibody to the COOH-terminal domain of p27 was determined by competition studies using excess peptide corresponding to amino acid residues 181–198 of human p27 (Fig. 2D). Together, the data indicate that first, a novel NH2-terminal truncated form of p27 is induced during cell cycle progression in NIH3T3 cells, and second, the appearance of this form of p27 coincides with increased DNA synthesis and expression of Cdc2, cyclin A, and cyclin B proteins.

Functional Role of Distinct Structural Domains of p27 in Regulation of CDC2 Kinase Activity—The members of the p21 family of Cdk inhibitors have a common amino-terminal region that inhibits the activity of Cdkks (16). In contrast, the carboxy-terminal domain of this family of Cdk inhibitors has evolved to become structurally distinct. Structure function analysis has provided evidence that aminoterminal domains of p21, p27, and p57 have Cdk-inhibiting properties (16). In contrast, the search for a common biological activity for the carboxy-terminal domains for these molecules has not been successful. Although, accumulating evidence indicates that the carboxy-terminal domain of p21 binds to proliferating cell nuclear antigen and inhibit its activity (16); as yet, no biological activity has been elucidated for the carboxy-terminal domains of p27 and p57. In view of our finding indicating that the appearance of a variant form of p27 consisting of the carboxy-terminal region correlated well with Cdc2 activity, we next sought to investigate a possible role for distinct structural domains of p27 in regulating Cdc2 activity in vitro. Accordingly, the carboxyl-terminal domain (Cp27, amino acids 101–198), amino-terminal domain (Np27, amino acids 1–100) and full-length p27 (amino acids 1–198) were bacterially expressed and purified (Fig. 3). The effects of purified recombinant p27 proteins on the activity of recombinant CDC2-cyclin B were examined by in vitro kinase assays using histone H1 as the substrate. As shown in Fig. 4A, full-length p27 exhibited a potent inhibitory effect on histone H1 phosphorylation by CDC2. As predicted, Np27 had a similar effect (Fig. 4B). In contrast, Cp27 unex-
expectedly stimulated the CDC2 kinase activity (Fig. 4C). When CDC2 kinase activity was assayed in the presence of different substrate concentrations, similar results were obtained (Fig. 4D). We observed a 5-fold induction in the maximal level of CDC2 kinase activity when incubated in the presence of Cp27. Together, these findings suggest that both p27 and Np27 contain the inhibitory region for CDC2 kinase activity, while the COOH-terminal region functions as a CDC2 activator.

Phosphorylation of p27 and Cp27 by CDC2 Kinase in Vitro; Localization of p27 Phosphorylation Site by CDC2 Kinase—During routine experiments to examine the effect of p27 on CDC2-cyclin B kinase activity, we also observed phosphorylation of the recombinant p27 and the COOH-terminal domain of p27 by CDC2 kinase. Under these conditions, the NH2-terminal domain was not phosphorylated by CDC2 (Fig. 5A). Since the carboxyl-terminal domain of p27 contains a consensus sequence for CDC2 kinase (amino acid residues 187) (14, 21, 22), we next sought to determine the phosphorylation site in p27 by CDC2 kinase. Accordingly, p27, and Cp27, which lacked a portion (amino acid residues 187–198) of the COOH-terminal domain, were engineered. The recombinant proteins, designated p27del and Cp27del, respectively, were bacterially expressed, purified, and tested for their ability to be phosphorylated by CDC2 kinase. As shown in Fig. 5A, both p27del and Cp27del failed to serve as a substrate for CDC2 kinase in vitro. These results indicate that p27 is phosphorylated within its carboxyl-terminal region (amino acid residues 187–198), which contains a consensus motif for CDC2 kinase.

Identification of the Role of Phosphorylation on Regulation of CDC2 Activity by p27—As the biochemical functions of many cell cycle cyclins are thought to be regulated by phosphorylation-dephosphorylation events (1), we next sought to investigate the role of p27 phosphorylation on its ability to modulate CDC2 kinase activity. As shown in Fig. 5B, loss of the phosphorylation site in wild type p27 did not affect its ability to inhibit CDC2 kinase activity. In contrast, deletion of the phosphorylation site from Cp27 completely abolished its activating effect on CDC2 (Fig. 5C). Together, these results make it possible to speculate that phosphorylation of p27 regulates only its activating, but not inhibitory, function. Further mutation analysis of putative phosphorylation site of p27 should allow us to investigate the role of phosphorylation in regulating its activating function.

Investigation of the contribution of p27 to cell cycle regulation has been limited to its role during the G2/M transition. p27 was first described as an inhibitor of cyclin E-Cdk2 in transforming growth factor-β-treated cells, which were contact-inhibited. Overexpression of p27 results in a G1 cell cycle arrest

In vitro, p27 inhibits cyclin E-Cdk2 kinase activity more effectively than other Cdk-cyclin holoenzymes, and this raises the question as to whether p27 may have differential specificity for regulating distinct cyclin-Cdk complexes (14, 24). The level of p27 is also regulated during the cell cycle. Accumulating evidence indicates that p27 reaches its maximal level in quiescent cells and gradually decreases as cells approach S phase, likely as a result of increased degradation through the ubiquitin-proteosome complex and the translational regulation of p27 mRNA (25).

Investigation of a possible role for p27 during the G2/M transition has been hampered by G1 arrest of cells overexpressing this molecule and the lack of a reliable inducible system to study the effects of this cyclin-dependent kinase inhibitors on the activity of different cyclin-Cdk holoenzymes. In the present report, we have taken advantage of the availability of recombinant p27 and CDC2-cyclin B complex to study the role of p27 in regulation of cell cycle progression through G2/M phase. Our results indicate that a variant form of p27, which lacks the NH2-terminal region, is expressed during G2/M, and that the expression pattern of this protein is temporally similar to that of CDC2 and cyclin B in vitro. We have shown that p27 regulates CDC2 kinase activity in vitro via an NH2-terminal inhibitory region and a COOH-terminal activating region.
Taken together, these findings suggest that regulation of p27 degradation and p27 phosphorylation play a major role in controlling cell cycle progression. Although the exact physiochemical properties of the variant form of p27 expressed in vivo remains unknown, our findings indicate that further investigation of the role of the carboxyl-terminal domains of p27 in regulating G2/M cell cycle progression is highly warranted.

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