Inactivation of the Cyclin D-dependent Kinase in the Rat Fibroblast Cell Line, 3Y1, Induced by Contact Inhibition*

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Cyclin-dependent kinase (Cdk) inhibitory proteins are involved in cell cycle arrest induced by antiproliferating factors or chemicals. High cell density also induces cell cycle arrest in which the genomic DNA is unreplicated, even in the presence of a mitotic dose of growth factors; this is termed contact inhibition. Although the cell cycle of the rat fibroblast cell line, 3Y1, was arrested in quiescence by contact inhibition, the Cdk4 bound to its regulatory subunit, cyclin D1 or D3. However, these complexes were enzymatically inactive. Phosphorylation of the cyclin D1-bound Cdk4 by the Cdk-activating kinase could convert the inactive cyclin D1-Cdk4 complex into its active form in vitro, suggesting that threonine 172 of the Cdk4, of which phosphorylation is required for its activation, was in part unphosphorylated in contact-inhibited 3Y1 cells. Although MO15 was active in cell extracts prepared from the arrested 3Y1 cells, activation of bacterially produced Cdk4 in the cell extracts was inhibited. Removal of p27kip1 from the cell extracts allowed the MO15 holoenzyme to phosphorylate the Cdk4 and in turn activate it, indicating that p27kip1 plays a role in inhibiting the phosphorylation of Cdk4 by MO15 in the contact-inhibited 3Y1 cells.

Sequential activation and inactivation of cyclin-dependent kinases (Cdks) govern the progression and the transitions of the cell cycle in eukaryotic cells. Their activity is regulated through phosphorylation of tyrosine or threonine residues and by the binding of inhibitors or regulatory subunits, called cyclins. In turn, the cyclins are regulated by their expression and degradation. In mammalian cells, as quiescent cells enter the cell cycle in response to growth factor stimulation, D-type cyclins (D1, D2, and D3) synthesized early in the G1 phase of the cell cycle form enzymatically active complexes with cyclin-dependent kinase 4 (Cdk4) or Cdk6 (1, 2). These complexes catalyze phosphorylation of the retinoblastoma gene product, pRb (2, 3), whose phosphorylation is necessary for cells to enter the S phase. Deprivation of growth factor during the G1 phase (2, 3), whose phosphorylation is necessary for cells to enter the S phase, whereas injection into G1/S transition cells shows no such effect (4, 5), suggesting that the cyclin D-Cdk4 complex exerts its effect during the middle to late G1 phase. To become a fully active holoenzyme, Cdk4 must bind a D-type cyclin as well as undergo phosphorylation at Thr-172 (6). The Cdk-activating kinase (CAK), composed of cyclin H and MO15 (alias Cdk7) (7–11), phosphorylates not only Thr-161 in Cdk2 and Thr-160 in Cdk2 (10, 11) but also Thr-172 in Cdk4 (12).

Cdk-inhibitory proteins (CKIs), induced by various antimitotic signals, prevent cells from progressing through the cell cycle; overexpression of the CKIs induces cell cycle arrest, suggesting that the induction of those CKIs triggers the arrest. Two families of CKIs have been identified in mammalian cells. The first family, called INK4, includes p16INK4a (13), p15INK4b (14), p18INK4C (15, 16), and p19INK4b (16, 17). It is composed of a repeat ankyrin-like sequence and selectively inhibits Cdk4 and Cdk6 by binding to the Cdk subunit alone. The second family includes p21cip1/waf1/sdi1 (18–22), p27kip1 (23, 24), and p57kip2 (25, 26), which can inhibit a broad range of cyclin-Cdk complexes. p21cip1 plays a physiologically important role in the regulation of cell proliferation in many tissues (27–29) and is induced by various antimitotic signals, such as transforming growth factor-β (30–32), cAMP (33), rapamycin (34), serum deprivation (35, 36), and contact inhibition (30). In quiescent cells, the level of p27kip1 protein is elevated. Accumulation of p27kip1 is correlated with inactivation of the G1 cyclin-Cdk complexes and results in G1 arrest. In cyclic AMP-treated macrophages, an increased amount of p27kip1 prevents the activation of the cyclin D-Cdk4 complex and induces G1 arrest (33). During T cell mitogenesis, antigen stimulation promotes the synthesis of cyclins and Cdks, after which interleukin-2 allows for the activation of the cyclin-Cdk complexes by decreasing the p27kip1 level. This is prevented by rapamycin (34). High cell density also induces cell cycle arrest with unreplicated genomic DNA. This phenomenon, termed contact inhibition, is generally observed in normal or untransformed cells, and loss of it is one of the remarkable properties of transformed cells. In the contact-inhibited rat fibroblast cell line, 3Y1, we showed that the cyclin D-dependent kinase was inactivated and that its inactivation was in part associated with the inhibition of the access of the Cdk-activating kinase to Cdk4 by p27kip1. During resumption of the G1 phase through the release of the cells from contact inhibition, we showed that timing of the activation of the cyclin D-dependent kinase correlated well with the reduction of the p27kip1.

EXPERIMENTAL PROCEDURES

Cell Culture and Cell Cycle Analysis—Exponentially growing 3Y1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 4 mM l-glutamine, and 40 μg/ml kanamycin. Confluent cells (described in our experiments as arrested cells by contact inhibition) were maintained for 4 more days.

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Cells released from the contact inhibition by trypsinization (growing cells) were diluted 7–10-fold with Dulbecco’s modified Eagle’s medium containing 10% FBS and maintained for 48 h. For analysis of the DNA content, monolayer cells were trypsinized and suspended in a 1-ml solution containing 0.1% sodium citrate, 0.1% Triton X-100, and 50 μM HEPES (pH 8.0), 0.5 μM NaF, 150 mM NaCl, 1% Nonidet P-40, and 0.25% sodium deoxycholate) containing 5 μg/ml of aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM NaF, 10 mM EGTA, and 2.5 mM MgCl₂, and assayed for pRb kinase activity. The cells described in Fig. 3d (0.5 × 10⁶ cells/lane) were lysed in CAK buffer, clarified by centrifugation, and the supernatants were mixed and incubated with the indicated amounts of recombinant His-tagged cyclin D2 or GST-Cdk4 in the presence of 1 mM ATP. The cyclin D2-Cdk4 complex bound to glutathione-Sepharose beads was collected by centrifugation and assayed for pRb kinase activity. The arrested cells described in Fig. 4b (0.5 × 10⁶ cells/lane) were lysed and immunodepleted by incubation with protein A-Sepharose beads precoated with saturating amounts of antiserum to p27kip1 or preimmune serum. Since GST-Cdk4 could be activated by the cyclin H-MO15 complex, we assumed that a major fraction of the bacterially produced GST-Cdk4 was denatured in the immune complex CAK assay was performed.

**RESULTS**

**Cyclin D-dependent Kinase Activity in 3Y1 Cells Arrested by Contact Inhibition**—In the presence of 10% FBS, the rat fibroblast cell line, 3Y1, grows exponentially and forms a monolayer on culture plates until the cells come in contact with each other. These cells then enter the quiescent phase of the cell cycle with no replication of the genomic DNA (Fig. 1a). This phenomenon is termed contact inhibition. When these arrested cells were released from their contact inhibition by trypsinization and then dilution in 10% FBS, the cells resumed their cell cycle progression. 48 h after the release, flow cytometric measurements of their DNA contents revealed growing cells in various phases of the cell cycle (Fig. 1a). Asynchronously growing 3Y1 cells expressed cyclin D1 and D3, and both of the D-type cyclins formed enzymatically active complexes with Cdk4 (Fig. 1, b and c). Although cyclin D1 and D3 were expressed and bound to Cdk4 in the arrested 3Y1 cells as well as in the asynchronously growing cells, neither of the complexes was active (Fig. 1, b and c).

Activation of the Cyclin D-dependent Kinase in Vitro by the Reconstituted Cyclin H-MO15 Holoenzyme—The cyclin D-Cdk4 complex is catalytically inactive until it undergoes phosphorylation at threonine 172 in the cyclin D-bound Cdk4 (6); this raises the possibility that phosphorylation of the Cdk4 could be inhibited in the arrested 3Y1 cells. Since the CAK, composed of cyclin H and MO15, is known to phosphorylate threonine 172 in the Cdk4 (12), we made polyclonal antibodies to cyclin H and MO15. Antiserum to MO15 precipitated the MO15 as well as the cyclin H-MO15 complex produced in insect cells (Sf9) infected with recombinant cyclin H and MO15 baculoviruses. The antiserum to cyclin H precipitated only cyclin H (Fig. 2a). Given the failure of the cyclin H antiserum to recognize the complex, the immune complex kinase assay was performed with the antiserum to MO15. The immunoprecipitated cyclin H-MO15 complex was subjected to the kinase assay with bacterially produced Cdk2 or Cdk4, fused to GST. GST-Cdk2 was phosphorylated by the cyclin H-MO15 complex immunoprecipitated with antiserum to MO15 (Fig. 2b). By contrast, GST-Cdk4 was not appreciably phosphorylated by the immunoprecipitated cyclin H-MO15 complex in both the absence and the presence of His-tagged cyclin D2 (data not shown). Since GST-Cdk4 could be activated by the cyclin H-MO15 complex as a pRb kinase (see below), we assumed that a major fraction of the bacterially produced GST-Cdk4 was denatured and that a minor fraction of the GST-Cdk4 was phosphorylated and activated by the cyclin H-MO15 complex.

To assess the activity of the cyclin H-MO15 complex pro-
Inhibition of Cdk4 Activation by p27kip1

In the absence of p27kip1, the inactive form of Cdk4 is converted into the active form as a complex with the cyclin H-MO15 holoenzyme only. To reduce endogenous CAK activity in a short period of time at a high temperature (see "Experimental Procedures"), we incubated the immunoprecipitated cyclin D-Cdk4 with insect cell lysates produced in Sf9 cells as CAK. These results suggest that the Cdk4 activation was in part suppressed in the contact-inhibited cells. Since it might be possible that Cdk4-CAK is reduced or inactive in the arrested cells, the CAK activity in contact-inhibited and growing cells was measured.

CAK Activity in 3Y1—When MO15 was precipitated from lysates of growing cells or from those arrested by contact inhibition, no significant differences in its Cdk2 kinase activities were detected (Fig. 3a). Moreover, immunoprecipitated MO15 from contact-inhibited cells as well as from growing cells could activate bacterially produced Cdk2 and Cdk4. Although the CAK activity was equally suppressed in contact-inhibited and growing cells, the activation of bacterially produced Cdk4 in contact-inhibited cells was lost, indicating that Cdk4 could not access CAK in the contact-inhibited cells.
Inhibition of Cdk4 Activation by p27kip1

more p27kip1 protein was present in the contact-inhibited cells than in the growing cells (Fig. 4a). To examine whether p27kip1 was involved in the inactivation of Cdk4 in contact-inhibited cells, p27kip1-deprived or nondeprived cell lysates were subjected to inhibition of the activation of bacterially produced Cdk4. Although the cell lysates were precleared with preimmune serum-coated protein A-Sepharose beads, somehow, a slight Cdk4 activation was observed, and the p27kip1 depletion allowed for an effective activation of Cdk4 in the contact-inhibited cell lysates (Fig. 4b). These findings indicate that p27kip1 was in part involved in the regulation of Cdk4 activation in which the MO15 cannot access Cdk4 in contact-inhibited cells.

When contact-inhibited 3Y1 cells were trypsinized and subsequently diluted 7–10-fold in the presence of a mitotic dose of serum (10% FBS), the cultured cells synchronously entered S phase 18–22 h later (Fig. 5c). Throughout the cell cycle, the protein levels of cyclin D1, Cdk4, and cyclin D1-bound Cdk4 did not fluctuate dramatically (Fig. 5a). As cells progressed toward the S phase, p27kip1 decreased and cyclin D-dependent kinase activity appeared. The timing of the activation of cyclin D-dependent kinase correlated well with the reduction of p27kip1, suggesting that p27kip1 also regulated cyclin D-dependent kinase activity in the cycling cells (Fig. 5, a and b).

**DISCUSSION**

Mammalian cells can enter the cell cycle in response to growth factor-induced signals. Although macrophages transiently deprived of colony-stimulating factor 1 halt in the early G1 phase and fibroblasts transiently deprived of serum halt in quiescence, both cells reenter the cell cycle upon restimulation by growth factors. When the growth of either cell types is arrested by deprivation of the growth factor, the expression of D-type cyclins is undetectable. However, fibroblasts constitutively expressing both D-type cyclins and Cdk4 undergo growth arrest by serum starvation. In these cells, overproduced D-type

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**Fig. 3. CAK activity in 3Y1.** Lysates of the growing 3Y1 (lanes 1–6), the arrested 3Y1 (lanes 7–12), or insect cells infected with MO15 virus together with cyclin H virus (lanes 13 and 14) were immunoprecipitated with nonimmune rabbit serum (lanes 6, 12, and 13) or antiserum to MO15 (lanes 1–5, 7–11, and 14) and then incubated with bacterially produced GST-Cdk2 in the presence of [γ-32P]ATP for the period of time indicated at the top (a). The precipitates from the arrested (lanes 1–5) or growing (lanes 6–10) cell lysates with nonimmune rabbit serum (lanes 1 and 6) or antiserum to MO15 (lanes 2–5 and 7–10) were incubated with the indicated combinations of bacterially produced cyclin A and Cdk2 in the presence of ATP. The GST-Cdk2, subsequently recovered on glutathione-Sepharose beads, was assayed for histone H1 kinase activity (b). Precipitates used in panel b were incubated with the indicated combinations of bacterially produced cyclin D2 and Cdk4 in the presence of ATP. The GST-Cdk4, subsequently recovered on glutathione-Sepharose beads, was assayed for pRb kinase activity (c). Lysates from the arrested or growing cells were incubated with the indicated amounts (μg) of bacterially produced His-tagged cyclin D2 and GST-Cdk4 for 1 h at 23°C in the presence of ATP. The recovered cyclin D2-Cdk4 complex on glutathione-Sepharose beads was assayed for pRb kinase activity (d).

**Fig. 4. Activation of cyclin D-Cdk4 complexes in the contact-inhibited cell lysates by removal of p27kip1.** Amounts of p27kip1 in the arrested or growing 3Y1 cells were analyzed by immunoprecipitation and subsequent immunoblotting with antiserum to p27kip1 (a). Extracts of the arrested 3Y1 cells (none) or cell extracts immunodepleted with nonimmune serum (pre-immune) or antiserum to p27kip1 (anti-KIP1) were mixed with both the immunoprecipitated cyclin H-MO15 complex and the bacterially produced cyclin D2 and Cdk4 in the presence of ATP for 1 h at 23°C and then assayed for CAK-mediated cyclin D2-Cdk4 activation (b).
cyclins and Cdk4 are detected even when the overexpressing cells are quiescent; however, the D-type cyclins and Cdk4 do not interact with each other (3). In contact-inhibited cells, in the presence of a mitotic dose of serum, the amounts of D-type cyclins and Cdk4 were identical with those of growing cells. Moreover, D-type cyclins bound Cdk4 despite the quiescence of the cells. Together, serum stimulation induced not only synthesis of D-type cyclins but also an assembly factor for D-type cyclins and Cdk4 even when the cells were in a quiescent state, whereas complexes composed of D-type cyclins and Cdk4 were enzymatically inactive in contact-inhibited cells. We reasoned that inhibition of Cdk4 phosphorylation by p27kip1 may play a crucial role in maintaining cell cycle arrest. As cells were released from the contact inhibition and progressed toward the S phase, the amounts of D-type cyclins, Cdk4, and their complexes did not fluctuate dramatically. p27kip1 decreased gradually, and then cyclin D-dependent kinase activity appeared. Timing of the activation of the cyclin D-dependent kinase correlated well with the reduction of p27kip1.

An increased amount of p27kip1 in contact-inhibited cells blocked the access of CAK to unphosphorylated cyclin D-bound Cdk4. The same manner of p27kip1-induced Cdk4 inactivation has been also observed in cAMP treated macrophages. The macrophage cell line, Bac1.2F5, requires colony-stimulating factor 1 to proliferate and is sensitive to cAMP-induced growth arrest. Cdk4 recovered from Bac1.2F5 arrested by cAMP treatment lacks threonine phosphate (33). These results suggest that inhibition of Cdk4 phosphorylation by p27kip1 may play a crucial role in maintaining cell cycle arrest. As cells were released from the contact inhibition and progressed toward the S phase, the amounts of D-type cyclins, Cdk4, and their complexes did not fluctuate dramatically. p27kip1 decreased gradually, and then cyclin D-dependent kinase activity appeared. Timing of the activation of the cyclin D-dependent kinase correlated well with the reduction of p27kip1.

An increased amount of p27kip1 is expressed in various cell lines arrested in quiescence by growth factor deprivation, such as colony-stimulating factor 1 in macrophages (33), interleukin-2 in T-cells (34), and serum in fibroblasts (35, 36). As these cells are released from quiescence by growth factor stimulation and cell cycle progression is resumed, the amount of p27kip1 gradually decreases through G1 phase. This reduction of the p27kip1 level supports the view that p27kip1 also regulates the kinase activity of cyclin D-Cdk4 complexes in cycling cells released from quiescence. D-type cyclins and Cdk4 are key regulators for G1 progression, and their kinase activities increase as cells approach the G1/S boundary.

In our experiments, p27kip1 plays an important role in the activation of Cdk4 by CAK during contact inhibition and in the regulation of cyclin D-dependent kinase activity in the G1 phase of cycling cells released from contact inhibition. Although p27kip1 can regulate a broad range of cyclin-Cdk complexes in vitro (23, 24), inhibition of Cdk4 may be one of the crucial roles for p27kip1 in cell cycle control as a Cdk inhibitor in contact-inhibited cells and in released cycling cells.

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REFERENCES

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