Activation of p27Kip1 Expression by E2F1
A NEGATIVE FEEDBACK MECHANISM*

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The E2F1 transcription factor is a critical regulator of cell cycle due to its ability to promote S phase entry. However, E2F1 overexpression also sensitizes cells to apoptosis and E2F1-null mice are predisposed to tumor development, suggesting that it also has properties of a growth suppressor. E2F1 transcription function is regulated by interaction with hypophosphorylated pRb. Cdk inhibitors such as p16INK4a and p27Kip1 inhibit pRb phosphorylation by the cyclin D/Cdk4 and cyclin E/Cdk2 complexes, thus keeping E2F1 in an inactive state. We found that E2F1 binds to the p27 promoter in vivo and activates p27 mRNA and protein expression. Depletion of endogenous E2F1 by siRNA causes a reduction in basal p27 expression level. Inhibition of endogenous p27 expression by siRNA increases E2F1 transcriptional activity and permits accelerated cell cycle progression by exogenous E2F1. These observations suggest that induction of p27 acts as a negative feedback mechanism for E2F1 and may also contribute to other functions of E2F1.

Progression of mammalian cells through normal cell cycle requires coordinated actions of positive and negative factors. Mitogenic signals stimulate sequential assembly and activation of cyclin D/Cdk4/6 and cyclin E/Cdk2 in early and late G1, resulting in the phosphorylation of pRb retinoblastoma protein and release of E2F transcription factors. The seven members of the E2F gene family play central roles in controlling S phase entry (1). Among the E2F proteins, E2F1, -2, and -3A promote cell growth, while E2F3B, -4, -5, -6, and -7 act as negative regulators (1, 2). E2Fs are inactivated by binding to the retinoblastoma family of pocket proteins. Hyperphosphorylation of pRb releases E2F1, which initiates transcription of genes required for G1/S transition, such as cyclin A, cyclin D, cyclin E, c-my c, c-my b, and DNA polymerase (1). Subsequent activation of cyclin E/Cdk2 and cyclin B/Cdk1 complexes are important for completion of mitosis.

The activities of cyclin/Cdk complexes are negatively regulated by Cdk inhibitors, including the INK4 family (p15INK4b, p16INK4a, p18INK4c, and p19INK4d) and Cip/Kip family (p21Cip1, p27Kip1, and p57Kip2). The INK4 family mainly inhibits cyclin D/Cdk4 complex and Cip/Kip family mainly targets cyclin E/Cdk2. p27 is regulated at the levels of transcription and protein turnover. Proteolysis by the ubiquitin-dependent prosome pathway is thought to be the predominant mechanism that regulates p27 levels. This occurs through phosphorylation at Thr187 by cyclin E/Cdk2 and subsequent recognition by ubiquitin ligase SCF Skp2 (3, 4). Cytoplasmic mislocalization of p27 may also lead to loss of function (5). In addition, p27 activity can be regulated by sequestration by cyclin D/Cdk4/6 complex, which prevents it from inhibiting cyclin E/Cdk2 (6, 7). Forkhead transcription factor has been shown to activate p27 transcription and induce cell cycle arrest (8, 9). Given its important roles in cell cycle regulation, p27 is likely to be controlled by multiple transcription factors that coordinate its expression with cell cycle progression.

Disruption of p27Kip1 locus in mice results in increased animal size due to increase in cell proliferation (10). Furthermore, p27Kip1-deficient mice also showed increased tumorigenesis after genotoxic or oncogenic insults (11, 12), suggesting that it can function as a tumor suppressor gene. Consistent with this notion, reduced levels of p27 expression are frequently observed in human carcinomas and correlate directly with poor prognosis (13). However, p27 has also been shown to promote assembly of active cyclin D/Cdk4 complex in vivo and is part of the active complex (14). Therefore, it is possible that p27 expression plays a role in coordinating cell cycle progression by increasing cyclinD/Cdk4 activity during early G1 phase.

Ectopic expression of E2F1 is sufficient to drive quiescent cells into S phase (15). As expected, E2F1 overexpression can cooperate with activated ras in inducing morphological and tumorigenic transformation of rat embryo fibroblasts in culture (16), which fits the classic definition of an oncogene. Interestingly, E2F1 also has activities that are opposite to its role as an oncogene. Overexpression of E2F1 in mouse embryo fibroblasts results in abortive S phase and significant apoptosis through p53-dependent and independent mechanisms (17, 18). E2F1-null mice develop testicular atrophy and exocrine gland dysplasia (19, 20), consistent with a growth suppressor function. Since formation of the pRb-E2F1 complex can lead to recruitment of histone deacetylases to E2F1 target promoters and actively repress transcription, loss of the repressive complexes in the E2F1-null mice may partially account for the cell proliferation phenotype. However, E2F1 has well documented target genes that are pro-apoptotic, such as caspase 3, 7, 9 and Apaf1 (21). Furthermore, E2F1 directly induces expression of p19ARF, which in turn activates p53 by binding and inhibiting MDM2 function (22). Therefore, the growth-promoting function of E2F1 is likely to be counterbalanced by multiple self-imposed safeguard mechanisms.

In this report, we present evidence that E2F1 can induce expression of p27 through activation of the p27 promoter. E2F1 expression is necessary for maintaining basal level p27 expression in the H1299 tumor cell line. Expression of p27 cooperates with pRb to suppress E2F1 activity, and ablation of endogenous p27 expression enhances E2F1 activity. The results suggest the

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presence of a novel p27-dependent negative feedback mechanism that regulates E2F1 activity.

MATERIALS AND METHODS

Cell Lines and Plasmids—H1299 (non-small cell lung carcinoma, p53-null), U2OS (osteosarcoma, wt p53), and NIH3T3 cells were maintained in DMEM medium with 10% fetal bovine serum. To inhibit p27 expression by RNA interference, double-stranded oligonucleotide (5'-GATCCTCCGTGACTAGAAGATCTCGGGTTGAGATATCCGACCGAGCACTTCTTACCTAACATTCTTTGTTA, p27 sequence underlined) was cloned into the pSuperiorRetroPuro vector (OligoEngine). The plasmid was packaged into retrovirus by transfection of the amphotropic packaging cell line LA (a kind gift from Dr. Pei Jing Sun, the Scripps Institute). H1299 cells were infected with the p27 shRNA retrovirus and selected with 1 μM puromycin for 10 days. Drug-resistant colonies were pooled for analysis. H1299 cells infected with a vector virus without the shRNA insert were also selected and pooled as control. Adenoviruses expressing GFP or E2F1 were amplified using 293 cells and cell lysate containing the viruses were used for the infection experiments.

Western Blot—Cells were lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) and centrifuged for 5 min at 10,000 × g, and the insoluble debris was discarded. Cell lysate (10–50 μg of protein) was fractionated by SDS-PAGE and transferred to Immobilon-P filters (Millipore). The filter was blocked for 1 h with phosphate-buffered saline containing 5% nonfat dry milk, 0.1% Tween 20. p27 was detected using a monoclonal antibody (Clone 57, BD Biosciences). E2F1 was detected using monoclonal antibody KH95 or rabbit polyclonal antibody C-20 (Santa Cruz Biotechnology). ARF was detected using 14PO2 antibody (NeoMarkers). The filter was developed using ECL-plus reagent (Amersham Biosciences).

RT-PCR Analysis and Chromatin Immunoprecipitation (ChIP)—Reverse transcription of total RNA was performed using the SuperScript III kit (Invitrogen). The following PCR primers were used: p27F (5'-TAAACCCGGACCTTGGAAGAG-3') and p27R (5'-GCTTCTTGGGCGTCTTGGC-3') to amplify a 450-bp product and actin F (5'-GCTCGTCGTCGAGATG-3') and actin R (5'-CAAACATGGTCGATGCACAAG-3') to amplify a 353-bp product. To avoid overamplification, samples were retrieved after 15, 20, 25, 30, and 35 PCR cycles and analyzed by agarose gel electrophoresis. Representative results are shown in Fig. 2C.

Chromatin immunoprecipitation was carried out using published procedure (23). Immunolabeled mouse embryonic fibroblast 10 (1) cells were transiently transfected with E2F1 expression plasmid (24). E2F1 IP was performed using KH95 antibody. Mock IP was performed with a GFP antibody. Coprecipitated chromatin was analyzed by PCR for the presence of p27 promoter DNA between −615 to −326 using p27PF (5'-CGCCTGTGTTATGAGTTGTTGGTCTTGTT-3') and p27PR (5'-GGAGGCTGAGGCAAGAAGAGAGATGAG-3') primers, generating a 294-bp product. Results at cycle 35 are shown in Fig. 2F.

Luciferase Reporter Assay—Cells (50,000/well) were cultured in 24-well plates and transfected with a mixture containing 10 ng luciferase reporter plasmid, 5 ng of CMV- lacZ plasmid, 20 ng of E2F1 expression plasmid, and 50 ng of pRB or p27 plasmid. Transfection was achieved using Lipofectamine PLUS reagents (Invitrogen), and cells were analyzed for luciferase and β-galactosidase expression after 24 h. The ratio of luciferase/β-galactosidase activity was used as an indicator of E2F1 transcription activity.

Immunofluorescence Staining and Cell Cycle Analysis—Cells cultured on chamber slides were transduced with E2F1 plasmid (24). E2F1 IP was performed using a monoclonal antibody (Clone 57, BD Biosciences). E2F1 was detected using a monoclonal antibody (Clone 57, BD Biosciences). E2F1 and SV40 large T-antigen expression plasmids and p27 expression level was examined by Western blot after 48 h. Ctrl, control. B, MCF-7 and NIH3T3 cells were transiently transfected with E2F1 and SV40 large T-antigen expression plasmids and p27 expression level was determined after 48 h. C, U2OS cells were transiently transfected with E2F1 expression plasmid and double stained for E2F1 (middle panel) and p27 (right panel) expression levels in the same cells.

RESULTS AND DISCUSSION

E2F1 Induces p27 Expression—In experiments aimed at studying the regulation of E2F1 transcriptional activity, U2OS and H1299 cells were transiently transfected with E2F1 expression vector. Western blot analysis showed reproducible increase of p27Kip1 expression level after E2F1 transfection, despite less than 50% of the cells were transfected in a population (Fig. 1A). Coexpression of the tumor suppressor pRB with E2F1 blocked p27 induction, consistent with the ability of pRB to inactivate E2F1. Additional experiments showed that E2F1 transfection also induced p27 expression in MCF-7 cells and the NIH3T3 mouse fibroblast (Fig. 1B). Furthermore, the SV40 large T-antigen, which inhibits pRB and activates endogenous E2F1, induced p27 expression in non-transformed NIH3T3 but not in MCF-7 cells (presumably already have activated E2F1 due to oncogenic transformation). To further confirm the ability of E2F1 to induce p27 expression, U2OS cells were transiently transfected with E2F1 plasmid and subjected to immunofluorescence double staining for E2F1 and p27. The results showed that cells expressing ectopic E2F1 had elevated levels of p27 staining (Fig. 1C). Although p27 induction correlated with E2F1 expression, the levels of p27 and E2F1 expression did not show strict correlation in the same cells (Fig. 1C). This discrepancy may be due to the fact that p27 level is also affected by other factors such as cell cycle stages.

To confirm the effect of transient E2F1 transfection on p27 expression, an H1299 cell line stably expressing E2F1-ERTM construct was treated with 4-hydroxytamoxifen to activate the E2F1 fusion protein (25). This treatment also resulted in significant increase of p27 expression level (Fig. 2A). In cells with activated E2F1, expression level of another E2F1 target p14ARF was also increased as expected (Fig. 2A). These results suggested that p27 expression is regulated by E2F1.

E2F1 binding to several promoters have been shown to inhibit gene expression during G1 and S, possibly due to recruitment of pRB and the associated histone deacetylase complexes (1). To determine the role of endogenous E2F1 in regulating p27 expression level, RNA interference targeting of E2F1 was employed. An H1299 cell line has been established that stably expresses E2F1 siRNA, resulting in −90% reduction of endogenous E2F1 level (27). Suppression of E2F1 expression by siRNA correlated with significant reduction of p27 level in these cells (Fig. 2B), despite similar rates of cell growth com-

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Footnote:

1 The abbreviations used are: GFP, green fluorescent protein; RT, reverse transcription; IP, immunoprecipitation; ChIP, chromatin IP; FACS, fluorescence-activated cell sorting; 4-OHT, 4-hydroxytamoxifen; siRNA, small interfering RNA.
pared with control H1299 cells. This result suggested that endogenous E2F1 is a major activator of basal p27 expression in H1299 cells under normal cell culture condition. Whether E2F1 or other E2F family members also negatively regulate p27 expression during growth arrest in culture, in vivo, or in tumors with reduced levels of p27 remains to be further investigated.

**Activation of p27 Promoter by E2F1**—The level of p27 can be controlled at the level of degradation. To examine the effect of E2F1 on p27 degradation rate, U2OS cells transfected with E2F1 were treated with cyclohexamide to block protein synthesis, and the rate of p27 turnover was examined by Western blot. No difference in p27 stability was observed (data not shown), suggesting that E2F1 may directly regulate p27 expression at the level of transcription. This mechanism was confirmed by semiquantitative RT-PCR analysis of H1299-E2F1-ER cells. Activation of E2F1-ER by 4-hydroxytamoxifen (4-OHT) resulted in significant increase in p27 mRNA level (Fig. 2C).

To determine whether E2F1 directly binds to the p27 promoter, chromatin immunoprecipitation was performed on mouse cells. PCR amplification of chromatin DNA cross-linked to E2F1 showed the presence of p27 promoter fragment containing the region from control cells, suggesting binding by endogenous E2F1. The signal was further increased when cells were transfected with E2F1 (Fig. 2F). Therefore, E2F1 may activate p27 transcription by interacting with the E2F1 binding elements on the promoter. Although mouse promoter sequences between H11002326 to H11002615 contain the sites for strong E2F1 response in plasmid reporter assays, upstream E2F1 sites may also play a role in a physiological context, since the longer promoter mutants were more active in reporter assays (Fig. 2E). Further experiments will be needed to determine the detailed mechanism of p27 regulation by E2F1 and other E2F family members in different phases of the cell cycle and in response to growth signals.

The transcriptional activity of E2F1 is controlled by pRb, which is in turn regulated by cyclin E/Cdk4-mediated phosphorylation. We hypothesized that E2F1 induction of p27 expression...
FIG. 3. A potential p27 negative feedback loop. A, activation of E2F1 leads to activation of p27 promoter and increased p27 expression. p27 inhibits cyclin E/cdk2 and cyclin D/cdk4 complexes, preventing phosphorylation of pRb. Hypophosphorylated pRb in turn binds to E2F1 and inhibits E2F1 activity. p27 induction by E2F1 may also have additional functions in positively or negatively regulating cell cycle progression. B, H1299 cells were transfected with the E2F1-responsive cyclin D3-luciferase promoter and pRb and p27 expression plasmids. Luciferase activity was determined after 24 h. Ctrl, control.

FIG. 4. Suppression of p27 expression enhances E2F1 activity. A, H1299 cells were stably transduced with retrovirus vector expressing p27 hairpin RNA. The suppression of p27 expression was confirmed by Western blot. B, H1299-p27Si cells with reduced p27 expression were transfected with p27 promoter-luciferase reporter and the indicated amounts of E2F1 expression plasmid. Luciferase activity was compared with H1299-Vector control after normalizing to cotransfected LacZ level. C, H1299-p27Si and H1299-Vector control cells were infected with identical concentrations of Ad-E2F1 or Ad-GFP adenoviruses (~50 plaque-forming units/cell) for 24 h, followed by addition of 100 ng/ml nocodazole for 18 h to inhibit mitosis. Cells were stained with propidium iodide and analyzed by FACS. Higher percentage of cells remaining in G1 phase indicates a reduced rate of S phase entry. Different shadings within the outlines of the FACS histograms are for quantitation purposes.
may form a negative feedback loop to limit the activity of E2F1 (Fig. 3A). To test whether this is the case, E2F1-responsive cyclin D3 promoter was used to test the effect of p27 expression on E2F1 transcription function. Coexpression of p27 or pRb each resulted in inhibition of E2F1 function (Fig. 3B). Furthermore, p27 and pRb cooperatively inhibited E2F1 function. In a different assay, Gal4-E2F1-CT fusion that contains the C-terminal transcription activation domain of E2F1 (304–437) was used to activate the Gal4-luciferase reporter. Coexpression of p27 and pRb also strongly suppressed the ability of Gal4-E2F1-CT to activate the Gal4 reporter (Fig. 3C). These results suggested that induction of p27 expression by E2F1 can potentially function as a negative feedback regulatory mechanism that limits E2F1 activity.

Inhibition of p27 Expression by siRNA Enhances E2F1 Functions—To further investigate the role of endogenous p27 in regulating E2F1 activity, p27 expression in H1299 cells was inhibited by retrovirus-mediated stable expression of a p27 shRNA (Fig. 4A). A stable pool of H1299 cells with ~90% reduction of p27 level (H1299-p27si) was transfected with p27-luc and E2F1 expression vector; activation of the luciferase reporters was consistently 2-fold stronger in p27-depleted cells (H1299-p27si) compared with control (H1299-Vec) (Fig. 4B). Similar effects were observed by transient transfection of p27 siRNA expression plasmid (data not shown). These results demonstrate that endogenous p27 plays a role in regulating the activity of the transfected E2F1.

E2F1 expression has been shown to promote cell cycle entry of quiescent cells (15). Induction of p27 expression by E2F1 may function in limiting the cell cycle promoting effect of E2F1. To test whether this is the case, H1299-p27si cells were infected with recombinant adenovirus vector expressing E2F1. Infected cells were analyzed for cell cycle progression by FACs after 24 h. To determine whether the ability of cells to enter and complete S phase was altered by E2F1 expression, cells were also treated with the microtubule-disrupting agent nocodazole for 18 h before harvest to prevent cells from passing through M phase. This assay enabled us to determine the rate by which G1 cells enter and progress through S phase, since G1 cells would not be replenished once entering S phase. Unlike quiescent cells, expression of E2F1 in control H1299-Vec cells did not have significant effects on the rate of cell cycle progression except inducing moderate levels of apoptosis (Fig. 4C). However, E2F1 clearly accelerated cell cycle progression in H1299-p27si cells, reducing the G1 population to ~65% of control level after the same duration of nocodazole treatment (Fig. 4C). These results suggested that p27 expression plays a role in restricting the cell cycle promoting activity of E2F1.

In summary, the data described above demonstrate that p27 is a transcriptional target of E2F1. Furthermore, endogenous E2F1 is an important factor in maintaining p27 basal expression level in H1299 cells. The ability of p27 to inhibit E2F1 activity in cooperation with pRb as demonstrated in this study and in previous publications, together with the accelerated cell cycle in p27-deficient cells upon E2F1 expression suggest the presence of a negative feedback loop that limits E2F1 activity (Fig. 3A). A recent study also showed that pRb/p27 double-null MEFs more readily escape crisis in culture and immortalize at a higher frequency than p27-null or pRb-null MEFs (30), consistent with the interpretation that p27 acts as a safeguard mechanism against pRb loss and E2F1 activation. Identification of the p27 negative feedback loop adds additional complexity to the cell cycle clock and reveals a new step that may be a target of growth signaling.

Our interpretation of the data does not exclude more complex roles of the E2F1-p27 connection. Contrary to its established function as a cell cycle inhibitor, p27 has also been shown to promote assembly of active cyclin D/Cdk4 complex in vivo and is part of the active complex (14). Therefore, it is possible that p27 induction by E2F1 plays a role in coordinating cell cycle progression by increasing cyclinD/Cdk4 activity during early G1 phase. Furthermore, E2F1-null mice are predisposed to tumor development (19, 20), suggesting that it also has properties of a growth suppressor. Induction of p27 may contribute to the tumor-suppressive function of E2F1 in vivo.

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