Regulation of the Expression of Cyclin-Dependent Kinase Inhibitor p21 by E2A and Id Proteins

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The helix-loop-helix transcription factor E2A plays important roles not only in promoting cellular differentiation but also in suppressing cell growth. Id proteins, the inhibitors of E2A, have opposite effects on cell differentiation and growth. To understand the mechanisms by which E2A suppresses cell growth, we examined the role of E2A in regulating the expression of the cyclin-dependent kinase inhibitor p21^{CIP1/WAF1/SDI1}, which prevents cell cycle progression upon overexpression. By using transient-cotransfection assays of luciferase reporter constructs in HeLa cells, we have found that overexpression of E2A can transcriptionally activate the p21 gene. To identify the sequences that mediate this activation in the promoter of the p21 gene, we carried out mutational analyses. Out of the eight putative E2A-binding sequences (E1 to E8) in the promoter, the E1 to E3 sequences located close to the transcription start site are found to be essential. In addition, loss of the E boxes in the promoter also reduces p21 expression without cotransfection with E2A in HIT pancreatic cells, where the endogenous E2A-like activity is high. Furthermore, we have also shown that overexpression of E2A in 293T cells activates expression of the endogenous p21 gene at both the levels of mRNA and protein. In correlation with the finding that E47 overexpression leads to growth arrest in NIH 3T3 cells, we have shown that Id1 overexpression in NIH 3T3 cells accelerates cell growth and inhibits p21 expression. Taken together, these results provide insight into the mechanisms by which E2A and Id proteins control cell growth.

The E2A transcription factor belongs to the basic helix-loop-helix (bHLH) family of proteins, which contains a conserved basic region responsible for DNA binding and a helix-loop-helix (HLH) domain for dimerization (24). The ubiquitously expressed E2A gene encodes two alternatively spliced products, E12 and E47, which differ in their bHLH domains and hence their DNA binding properties (24, 38). E47 homodimers bind to the E-box sequence (CANNTG) with a much higher affinity than E12 homodimers (41). The DNA binding activity of E2A proteins can be abolished by the HLH Id proteins (Id1 to -4) that serve as dominant-negative inhibitors (5, 7, 30, 39). The Id proteins dimerize with the E2A proteins, but the heterodimers cannot bind to DNA because Id proteins lack the DNA binding domain. Consequently, the Id and E2A proteins play opposing roles in regulating cell differentiation and proliferation. Because the expression of Id genes is generally downregulated during cell differentiation and restricted in the G1 phase of the cell cycle, the function of constitutively expressed E2A proteins may be modulated by altering the levels of the Id proteins.

As a stimulator of cell differentiation, E2A has been found to dimerize with tissue specifically expressed bHLH proteins and to activate specific gene expression, leading to the differentiation of various cell types including muscle, neuronal, and pancreatic cells (22, 25, 42). E2A proteins have also been shown to exist as homodimers in B lymphocytes (2), and null mutation of the E2A gene in mice results in severe defects in early B-cell development (3, 44). In contrast, the Id proteins have been shown to act as inhibitors of cell differentiation. Overexpression of the Id genes inhibits the differentiation of C2C12 myoblasts, erythroleukemia cells, and mammary epithelial cells (9, 18, 34). Furthermore, constitutive expression of Id1 in B lymphocytes blocks early B-cell development in transgenic mice (37).

In regulating cell proliferation, E2A was found by Peverali et al. to cause growth arrest in NIH 3T3 cells (28). When E2A was overexpressed in NIH 3T3 cells, the efficiency of colony formation was dramatically reduced, an effect similar to that caused by the myogenic bHLH protein MyoD (35). Moreover, it has been demonstrated that the E2A-induced growth arrest occurs before the G1 to S transition in the cell cycle (12, 33). Expression of p21 is activated upon inhibition of kinase activities, which are essential for all phases of cell cycle transition (12, 33). Expression of p21 has been shown to bind to the cyclin-CDK complexes resulting in the inhibition of kinase activities, which are essential for all phases of cell cycle transition (12, 33). Expression of p21 is activated by p53 (11), and activation of p21 expression results in cell cycle arrest at the G1 to S phase transition (32). Similar to transforming growth factor β (8), c-EBPα (40), and MyoD (13), E2A stimulates p21 expression independently of the p53-binding sites located in the p21 promoter. We have further demonstrated that the transcriptional activation by E2A is
mediated through the E-box sequences in the p21 promoter. Using Id1-inducible cell lines, we have shown that overexpression of Id1 can inhibit p21 expression and accelerate cell growth. Taken together, these results suggest that p21 may serve as a potential target through which E2A and Id proteins control cell proliferation.

MATERIALS AND METHODS

Plasmid construction. The p21P-luc construct (a gift from X.-F. Wang, Duke University) contains the 2.4-kb HindIII fragment of the p21 promoter inserted into the 5.2-kb EcoRI-HindIII fragment of pBluescript II (Stratagene, La Jolla, Calif.) (9). The p21P-luc construct was produced by digesting the p21P construct with SacI and HindIII and inserting the 2.4-kb SacI-HindIII fragment into the Smal- and HindIII-digested pGL2-basic vector. The p21PAPs construct containing an internal deletion in the promoter was generated by cloning the XhoI-PvuII and Smal-HindIII fragments into the XhoI and HindIII sites of the pGL2-basic vector. To construct p21PAPSm1, p21PAP was digested with PvuII and religated. The p21PAPSm2 construct was made by digesting p21PAP with PvuII and inserting an Nhel linker.

Successive mutations of the E boxes from E1 to E8 were introduced by using a two-step PCR procedure (41) with specific primers for each E box that change the CAGNTG sequence to CAGNAT (see Fig. 2A). To generate p21PE1-2, a PCR reaction containing the promoter sequence was synthesized using a primer, 5'-pGL, that binds to the sequence upstream from the polylinker of the pGL2 vector and a 3' primer, 3'-Nhe, that includes the sequence upstream from the E2 box plus the Nhel linker sequence. The p21P-luc plasmid was used as a template for the PCR product and was then digested with XhoI, Nhel, and cloned into the p21PAPSm2 construct through a three-part ligation with the XhoI-EcoRI and EcoRI-Nhel fragments from p21PAPSm2. To create p21PE1-3, a similar XhoI-Nhel fragment containing the E3 mutation generated by using two-step PCR with specific primers for each E box was then inserted into the 5'-pGL and 3'-Nhe primers. The template for each construct was the p21P construct that contains one less E-box mutation than the desired construct, e.g., the template for the p21PE1-5 construct was p21PE1-4. The PCR products were digested with XhoI and PstI and inserted into the XhoI (in the polylinker) and PstI (located between the E3 and E4 boxes) sites of p21PE1-3. To generate p21PE1-2, which contains point mutations of the E1 and E2 boxes rather than a Nhel linker insertion, specific primers together with the 5'-pGL primer and a 3' primer, 3'-Luc, that binds to the luciferase sequence were used in a PCR with p21P as a template. The PCR product was digested with PstI and HindIII and inserted into p21P through a three-part ligation. All E-box mutations were identified by the loss of PvuII sites in the wild-type sequence or the gain of a BamHI or EcoRI site included in the primers, as indicated in Fig. 2A.

To construct the pOP3-Id1 expression plasmid, the pOP3CAT vector was modified by replacing the NotI fragment containing the CAT gene with a pair of polylinker oligonucleotides that destroyed the NotI site at the 5' end of the insert and added XhoI and ApaI sites upstream from the 3' NotI site. A XhoI-NotI fragment containing the Id1 cDNA fused with the sequence encoding the hexamer oligonucleotides. The cDNAs were then amplified by PCR with p21- and E47-specific primers. These primers were as follows: 5'-ATGGCGGGCTGTC-3' and 5'-GTCTGC 3' for p21P and p21P2 constructs, respectively.

Transient transfection and assay of the luciferase and b-galactosidase activities. Transiently expressed E47 protein induces the p21 promoter. MyoD has previously been found to stimulate p21 expression in C2C12 myoblasts (13). We reasoned that MyoD might form heterodimers with E2A and directly activate the p21 gene. If so, the E2A gene products as homodimers may also be able to activate p21 expression in nonmuscle cells. To test this hypothesis, we cotransfected a luciferase reporter construct driven by a 2.4-kb promoter sequence of the p21 gene (p21P-luc) with an expression plasmid producing one of the E2A gene products, E47. As shown in Fig. 1, overexpression of E47 increased the p21 promoter activity by 19-fold in HeLa cells. Similarly, the p21P2 construct was activated by 21-fold, suggesting that the activation by E47 is independent of the p53 binding sites that are deleted in p21P2. E47 also activated the expression of the p21P-luc construct by 20-fold in NIH 3T3 cells (data not shown).

A computer search of the 2.4-kb promoter sequence of the p21 gene revealed eight putative E-box sites (Fig. 1). To identify the E-box sequences that might mediate the transcriptional activation by E47, we initially created an internal deletion construct, p21PAPSm-luc, which removed the six distal E boxes (E3 to E8) located between -60 and -1350 but retained the two proximal E boxes (E1 and E2) situated between the TATA box and the initiation site of transcription. The E1 sequence (CCAGCTG), located immediately upstream from the transcription start site, belongs to the E boxes (group I) that are avidly bound by E47 homo- or heterodimers, whereas the E2 sequence (CCAGCTGG) is a member of the E box (group III) that have much less affinity to E47 (38). The p21PAPSm-luc construct was transfected with or without the E47 expression plasmid into HeLa cells, which have very low levels of endogenous E47 or E47-like activity. In the absence of E47, this construct is expressed at a lower level compared to the wild-type construct p21P-luc, probably due to the deletion of the internal fragment that might contain binding sites for constitutively active transcription factors, e.g., Sp1. Despite the lower
basal activity, expression of this construct was stimulated 27-fold upon cotransfection with E47, suggesting that the E1 and E2 sequences may be responsible for the transcriptional activation by E47. To further demonstrate that the two E boxes can mediate the activation by E47, mutations were generated in the context of p21PΔPS-luc. In construct p21PΔPSm1, the 15-bp sequence from the E1 to E2 boxes was deleted, which recreates the E1 box in the reverse orientation. In construct p21PΔPSm2, both E1 and E2 boxes were destroyed by a linker insertion. Although the transcriptional activity of these two constructs did not alter dramatically in the absence of E47, E47-mediated activation was reduced to 15-fold for p21PΔPSm1 and 5-fold for p21PΔPSm2. The fivefold activation in construct p21PΔPSm2 is probably mediated by cryptic E-box sequences present in the luciferase vector because several unrelated luciferase constructs with minimal promoter sequences without E boxes display similar degrees of activation in the presence of E47 (data not shown). These results suggest that at least one of the E-box sequences located between the TATA box and the transcription start site is required for the transcriptional activation of the p21PΔPS construct by E47 in HeLa cells.

To further evaluate the role of the eight E boxes in activating p21 gene expression, we introduced successive mutations in the E boxes without gross alterations of the promoter sequence (Fig. 2A). In construct p21PE1-2, the E1 and E2 boxes were destroyed by a linker insertion, as in p21PΔPSm2. p21PE1-2 was then used to generate subsequent constructs in which additional E boxes were mutated from CAGNTG to ATGCTG and CAGCAT, respectively (Fig. 2A), without altering the sequence between the two E boxes. The resulting construct, p21PE1-2, behaved similarly to construct p21PE1-2, in which the E1 and E2 boxes were destroyed by a linker insertion (Fig. 2B). These results further support the conclusion that E47 activates p21 expression through the E boxes present in the promoter of the p21 gene. It is particularly interesting that the E1 and E2 boxes, located between the TATA box and the transcription start site, play a major role in the transcriptional activation by E47. The E boxes present upstream from the TATA box appear unable to substitute for the function of E1 and E2 boxes.

**E-box-mediated activation of p21 transcription in HIT cells.**

To determine the role of the E boxes in stimulating p21 expression in the absence of exogenous E47, we examined the expression of p21 promoter-reporter constructs in HIT pancreatic cells, which, unlike HeLa cells, are known to have E-box-mediated activating activities (25). We transfected the p21PΔPS and p21PΔPSm2 constructs (Fig. 1) with or without a vector expressing Id1. The relative luciferase activities are shown in Fig. 3. When construct p21PΔPS, which contains wild-type E1 and E2 boxes, was cotransfected with the Id1-expressing plasmid, its activity was reduced by 51%. Because Id1, as an inhibitor, may diminish E-box-mediated activation by E47 homodimers or heterodimers with pancreatic β-cell-specific bHLH proteins like BETα2, the inhibition of p21PΔPS expression by Id1 may indicate the extent of contribution by endogenous bHLH proteins to the expression of construct p21PΔPS. In comparison to p21PΔPS, 62% of the luciferase activity was observed with construct p21PΔPSm2, which carries E1 and E2 mutations. Furthermore, cotransfection of Id1 did not significantly reduce the activity of p21PΔPSm2. These results suggest that the activation of p21PΔPS expression by endogenous bHLH proteins in HIT cells is primarily mediated through the E1 and E2 boxes, which were found to be essential for E47-activated expression in the context of the full-length promoter of the p21 gene (Fig. 2).

**E47 activates endogenous p21 expression.** To test if E47 could stimulate expression of the endogenous p21 gene in
addition to the p21 promoter-reporter constructs, we transfected the expression plasmid producing the full-length E47 into 293T cells, which can be transfected at a high efficiency. The transfected cells were used to prepare cytoplasmic and nuclear fractions, and the fractions were analyzed by Western blotting. As shown in Fig. 4A, the E47 protein is present primarily in the nuclear fraction. The same blot was then probed with antibodies against p21. Comparing the cells transfected with the E47 expression construct to the vector control, the level of p21 protein is dramatically increased. The level of p21 mRNA, as determined by Northern blotting, was also increased by about fourfold in cells transfected with E47 (Fig. 4B). However, it remains to be determined whether the increase in p21 mRNA can be entirely attributed to the increase in p21 protein or whether additional posttranscriptional regulatory mechanisms also exist. Nevertheless, these results confirm the stimulatory effect of E47 on p21 expression.

Expression of Id1 accelerates cell proliferation. Since E47 has been shown to induce growth arrest when overexpressed in NIH 3T3 fibroblasts (28), we set out to perform a reciprocal experiment to test if overexpression of the inhibitors of E47, the Id proteins, could stimulate cell growth. To do so, we generated NIH 3T3 cell lines expressing Id1 under the control of an inducible promoter in the pOPI3-CAT vector (Stratagene). The coding region of Id1 containing a C-terminal fusion with the sequence encoding the HA epitope tag was inserted into the pOPI3-CAT vector to replace the CAT gene. The pOPI3-Id1 plasmid was cotransfected with the Lac repressor-expressing vector so that expression of Id1 was inhibited by the repressor in the absence of IPTG. After selection, several dozens of colonies carrying either the pOPI3-Id1 or pOPI3-CAT construct were obtained and expanded. To evaluate the level of expression and the inducibility of these clones, Western blotting was performed with a monoclonal antibody against the HA epitope. Clones Id1-12, Id1-172, and Id1-176 were found to express Id1 at much higher levels in the presence of IPTG under several culture conditions (Fig. 5). These clones and two CAT-expressing control clones, CAT-3 and -8, were used in further studies.

To monitor the growth properties of these cell lines, we have plotted growth curves for the three Id1 cell lines and the two CAT control cell lines grown with or without the IPTG inducer in the presence of 1, 2, or 5% fetal calf serum. The results of one such experiment are shown in Fig. 6. Induction of CAT
expression with IPTG at any serum concentration did not result in any increase in the growth rate of the CAT-3 and CAT-8 cell lines. At low serum concentrations, it actually slowed down the growth rate of the CAT-3 cells. In contrast, induction of Id1 expression in the Id1-12, Id1-172, and Id1-176 cell lines led to a modest increase in the growth rate under all culture conditions. For the Id1-176 cell line, an even more dramatic acceleration was observed when cultured in the presence of 1% fetal calf serum. These results suggest that overexpression of Id1 stimulates the growth of NIH 3T3 cells.

Transcriptional inhibition of the p21 gene by Id1. Since E47 was capable of inducing p21 expression, it might be possible that the accelerated growth in the Id1-overexpressing cell lines was due to the inhibition of E47 activity and hence the reduction of p21 expression. The p21 mRNA levels in the above cell lines with and without induction by IPTG were determined by RT-PCR assays (Fig. 7A). The Id1-expressing cell lines when induced by IPTG had lower levels of p21 mRNA (two- to threefold) than their noninduced counterparts. In contrast, the control cell lines CAT-8 and CAT-3 when induced by IPTG expressed slightly higher levels of p21 mRNA than their noninduced counterparts.

Consistent with the finding shown in Fig. 7A, when the p21 promoter-luciferase reporter construct (p21P-luc) was transiently transfected into the Id1-expressing and control cell lines, a reduction in luciferase activity was also observed upon induction for Id1 expression but not for CAT expression (Fig. 7B). Taken together, these data suggest that Id1 overexpression leads to the reduction of p21 expression, which may contribute to the increased growth rate of Id1-expressing NIH 3T3 cells.

**DISCUSSION**

The bHLH proteins play a fundamental role in eukaryotic cell differentiation and proliferation. The bHLH E2A transcription factor, like its dimerization partner, MyoD, has been shown to cause growth arrest in NIH 3T3 cells (28, 35). This function of E2A or MyoD is consistent with its role in stimulating cell differentiation. Growth arrest is usually associated with terminal differentiation. For example, myoblasts can be induced to undergo terminal differentiation by serum withdrawal in culture. In this process, expression of the Id genes is downregulated (5), which may allow the preexisting E2A and MyoD proteins to activate muscle-specific gene expression as well as the expression of genes that lead to growth arrest, e.g., the p21 gene. Expression of the p21 gene is indeed shown to be activated during the differentiation of various cell types (13, 19, 27). Although it is convenient to use this paradigm to explain the differentiation of myoblasts, preadipose cells, or osteoblasts, the situation may be more complicated during the differentiation of hematopoietic cells, which differentiate long before growth arrest. For instance, it is clearly demonstrated that the differentiation of progenitor B lymphocytes depends on the E2A protein (3, 44), probably for its ability to transcriptionally activate B-cell-specific genes. Why isn’t the growth of B cells arrested by the E2A proteins? In fact, continued proliferation of the differentiating cells is essential for the development of the B-cell lineage. Perhaps there are additional control mechanisms that prevent E2A from activating p21 expression, or the activation by E2A is insufficient in the absence of other transactivators, or the mitogenic signals for B-cell proliferation can override the effects of growth suppressors temporally or constitutively.
We have demonstrated here that just like MyoD (13), E2A can activate transcription of the p21 gene. Since MyoD forms heterodimers with E2A in vivo (20), and the heterodimers have a similar DNA binding specificity as the E2A homodimers (6, 38), it would not be surprising that both the E2A homodimers and heterodimers activate p21 expression by a similar mechanism. A straightforward mechanism would be that these homo- or heterodimers bind to the E-box sequences present in the promoter of the p21 gene and directly activate transcription. We have shown that the three proximal E boxes are important for p21 expression. In particular, the E1 and E2 boxes contribute to about two-thirds of the transcriptional activation by E47 in HeLa cells. Intriguingly, this activity mediated by the E1 and E2 boxes cannot be substituted by the E boxes present upstream of the TATA box. Because of the unique location of the E1 and E2 boxes, i.e., in the region between the TATA box and transcription start site, it is possible that these E boxes bring E47 to a close proximity with the basal transcription machinery and thus enable E47 to activate transcription more efficiently. Alternatively, E47 may cooperate with other transcription factors that bind to sequences adjacent to the two E boxes to activate transcription. In support of the functional significance of the E1 and E2 boxes, one E box in a similar location can also be found in the promoters of the mouse and rat p21 genes. Our data obtained with construct p21PAPSm1 suggested that even one E box in this location can activate a substantial level of p21 transcription. The E3 box located 130 bp upstream from the TATA box also contributes to the activation of p21 expression, but the E4 to E8 boxes have no effect on p21 expression even when the E1 to E3 boxes are mutated. The inability of the distal E boxes to activate p21 expression may suggest that E boxes cannot function at a distance unless they reside in enhancers consisting of multiple transcription factor binding sites, e.g., the enhancers of immunoglobulin genes (36). Halevy et al. have demonstrated that transcriptional activation of the p21 gene by MyoD occurs in p53-deficient mouse embryonic fibroblasts and in the absence of the p53 response element in the promoter of the p21 gene (13). We have shown here that E47 can also activate p21 expression without the p53 response element, suggesting a mechanism independent of p53.

The Id proteins, whose expression is induced by serum and restricted in the G1 phase of the cell cycle (4, 14, 29), have opposite effects on cell growth compared to the E2A or MyoD proteins. Incubation of NIH 3T3 cells with antisense oligonucleotides against Id transcripts blocks the cell cycle at the G1 to S phase transition (4, 14), suggesting that the Id proteins are required for cell cycle progression. Coexpression of Id1 with E47 reverses the growth arrest by E47 in NIH 3T3 cells (28). We have overexpressed Id1 using an inducible system and shown that Id1 is able to accelerate the growth of NIH 3T3 cells. This effect of Id1 could be, at least in part, attributed to the inhibition of p21 expression. We have found that in the Id1-expressing cell lines expression of either the endogenous p21 gene or the p21 promoter-reporter construct is reduced by two- to threefold. This consistent but modest reduction in p21 expression is probably due to the intrinsic low levels of E47 activity in NIH 3T3 cells. In contrast, overexpression of E47 leads to a dramatic increase in p21 expression as found in 293T cells. It would be interesting to determine the threshold of p21 concentration that influences the growth property of NIH 3T3 cells. As shown here, a reduction of p21 expression by two- to threefold could result in a modest stimulation of growth in NIH 3T3 cells. A more profound increase in p21 expression caused by overexpression of E47 may lead to growth arrest of the cells. Furthermore, in senescent human diploid fibroblasts, p21 expression is found to be elevated while Id1 and Id2 expression are downregulated (10, 26). This inverse relationship might be explained by the activation of p21 expression by E2A-like proteins, whose activities are inhibited by Id proteins in presenescent states. Overexpression of Id1 can indeed reactivate DNA replication of senescent human fibroblasts when coexpressed with a mutant of simian virus 40 T antigen defective in pRb binding (15). Although activation of p21 expression may be one mechanism by which E47 arrests cell growth, it remains to be determined if additional growth-suppressing genes are also activated by E47.

Could the Id proteins stimulate cell growth by other mechanisms independent of the function of E2A? Id2, but not Id1 or Id3, has been shown to bind to the Rb family of proteins (17, 21). By doing so, it alleviates the growth suppression caused by
FIG. 6. Growth curves of the Id1-expressing and control cell lines. The indicated cell lines (right) were cultured in the presence of a 1% (A), 2% (B), or 5% (C) concentration of fetal calf serum. To induce expression, IPTG was added 10 h before seeding the cells.
the expression of Rb, p107, or p130 in pRb-negative cells such as the Saos-2 osteosarcoma cells. Interestingly, Id2, but not Id1 or Id3, can also reverse the cell cycle arrest induced by overexpression of CDK inhibitor p16 or p21 (21). It was proposed that these CDK inhibitors prevent the phosphorylation of the Rb family of proteins and hence their inactivation. The binding of Id2 to unphosphorylated pRb or its relatives directly activates these proteins and therefore bypasses the regulatory mechanism mediated by the CDKs. Since this function of Id2 appears to be independent of the bHLH E proteins, it is not surprising that Id1 and Id3 do not have this effect. It does not exclude the possibility that Id2 could also diminish the expression of the CDK inhibitors by inhibiting the E proteins.

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