Id2 Specifically Alters Regulation of the Cell Cycle by Tumor Suppressor Proteins

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Cells which are highly proliferative typically lack expression of differentiated, lineage-specific characteristics. Id2, a member of the helix-loop-helix (HLH) protein family known to inhibit cell differentiation, binds to the retinoblastoma protein (pRb) and abolishes its growth-suppressing activity. We found that Id2 but not Id1 or Id3 was able to bind in vitro not only pRb but also the related proteins p107 and p130. Also, an association between Id2 and p107 or p130 was observed in vivo in transiently transfected Saos-2 cells. In agreement with these results, expression of Id1 or Id3 did not affect the block of cell cycle progression mediated by pRb. Conversely, expression of Id2 specifically reversed the cell cycle arrest induced by each of the three members of the pRb family. Furthermore, the growth-suppressive activities of cyclin-dependent kinase inhibitors p16 and p21 were efficiently antagonized by high levels of Id2 but not by Id1 or Id3. Consistent with the role of p16 as a selective inhibitor of pRb and pRb-related protein kinase activity, p16-imposed cell cycle arrest was completely abolished by Id2. Only a partial reversal of p21-induced growth suppression was observed, which correlated with the presence of a functional pRb. We also documented decreased levels of cyclin D1 protein and mRNA and the loss of cyclin D1-ckd4 complexes in cells constitutively expressing Id2. These data provide evidence for important Id2-mediated alterations in cell cycle components normally involved in the regulatory events of cell cycle progression, and they highlight a specific role for Id2 as an antagonist of multiple tumor suppressor proteins.

Undifferentiated cells must overcome growth-inhibitory signals to maintain their proliferative potential, which requires the inactivation of negative regulators of cell cycle progression such as the retinoblastoma protein (pRb) (68) and the related proteins p107 (21, 72) and p130 (28, 41). These proteins share a stretch of homologous sequence, called the pocket, that is required to bind other cellular proteins, including E2F (6, 9, 31, 66), cyclins (16, 20, 22, 28, 40, 41), and myoD (24). The interaction between pRb family members and these proteins results in the timely transcription of genes encoding factors essential for cell cycle progression (E2F) or induction of the differentiated state (myoD).

Several lines of evidence indicate that pRb or a related pocket protein is required for differentiation of a variety of cell types. pRb levels markedly increase upon differentiation of basal keratinocytes and colonic crypt cells (63). Introduction of pRb into pRb-negative Saos-2 cells allows the expression of muscle-specific genes in response to myoD (24). Loss of the RB gene or functional inactivation of proteins of the pRb family by viral oncogenes blocks differentiation and prevents the normal G0 arrest observed in differentiated skeletal muscle (7, 24, 57). p107 has been shown to substitute for pRb function in inducing growth arrest and differentiation in myoblasts derived from mice genetically deficient in RB. However, permanent withdrawal from cell cycle requires pRb function and is a prerequisite for terminal differentiation of myoblast cells (57).

In normal cells, pRb activity is regulated by alternating changes in pRb phosphorylation (5, 10, 15, 23). G1 cyclins functioning as regulatory subunits of their partners cyclin-de-
to 106) and Id3 (codons 40 to 81) were obtained by PCR amplification and full-length coding region of human Id1 (73) and mouse Id3 (11). To generate described in a previous report (34). GST-Id1 and GST-Id3 (31a) included the Id2 (a GST-Id2 fusion protein including only the HLH region of Id2) were fusion protein in which the HLH region of Id2 has been deleted), and GST-HLH and G418. The cells were plated 24 h prior to transfection in 100-mm-diameter calcium phosphate method. Forty-eight hours after transfection, the cells were split 1:4, and rabbit antiserum against Id1 (1:250) (35) or Id2 (1:50) (34) or normal rabbit serum were found to vary from 80 to 130 per plate. The effect of p16 and p21 expression were analyzed under a Zeiss fluorescence microscope. Approximately 200 phycoerythrin-positive cells were counted at daily intervals by hemocytometer, and cell viability was determined by trypan blue dye exclusion.

In vitro binding assay. 35S) methionine-labeled proteins were prepared in vitro, using a reticulocyte lysate system (Promega). Plasmids for in vitro trans- lation were prepared as previously described (35). Translation reactions were carried out essentially as previously described (54). Pools of U2OS-vector, Saos-2, and U2OS cells were transfected as previously described (34).

S-phase analysis and colony-forming efficiency assay. S-phase evaluation of U2OS, Saos-2, and U2OS cells was performed as previously described (34). Briefly, cells were transfected by the calcium phosphate method with pCMV- CD20 DNA, encoding the cell surface marker CD20 (72), and the plasmids indicated. Forty-eight hours after removal of DNA precipitates, bromodeoxyuridine (BrdU) was added to the cell culture medium to a final concentration of 10 μM for 2 h. Cells were doubly stained with a phycoerythrin-conjugated monoclonal antibody to BrdU (Boehringer Mannheim) and a fluorescein-conjugated monoclonal antibody to BRD (Boehringer Mannheim) and analyzed under a Zeiss fluorescence microscope. Approximately 200 phycoerythrin-positive cells were counted, and the fraction of cells that were also positive for fluorescein isothiocyanate was determined for each sample. The colony formation assay was carried out essentially as previously described (54). Pools of U2OS-vector, U2OS-Id1, and U2OS-Id3 (Table 1, Results) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and G418. The cells were plated 24 h prior to transfection in 100-mm-diameter dishes at a density of 104 per dish. Samples (20 μg) of recombinant plasmid DNA were dissolved in 30 μl of Glutathione S-transferase (GST)–Id2, GST-Id1, and pGEX-2T (Pharmacia). Immunoprecipitation, Western blotting (immunoblotting), and coimmuno- precipitation proteins were boiled in 2% SDS to dissociate complexes and diluted in SDS-gel loading buffer, de- natured in SDS-gel loading buffer, fractionated by electrophoresis on an SDS- 15% polyacrylamide gel, and visualized by autoradiography. To analyze cyclin D1-cdk4 complexes, U343 transfectants (107) were lysed in 1 ml of NP-40 buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40 [NP-40], 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing 1 mM phenylmethylsulfonyl fluoride and 10 μg each of aprotonin and leupeptin per ml. Lysates were clarified by centrifugation, and aliquots of the supernatants (0.5 ml) were incubated with antiserum against Id1 (1:250) (35) or Id2 (1:50) (34) or normal rabbit serum (NRS). After incubation with protein A-Sepharose beads (Santa Cruz Biotech- nology), the precipitates were washed three times in RIPA buffer, de- natured in SDS-gel loading buffer, fractionated by electrophoresis on an SDS- 15% polyacrylamide gel, and visualized by autoradiography. To analyze cyclin D1-cdk4 complexes, U343 transfectants (107) were lysed in 1 ml of NP-40 buffer (50 mM Tris-HCl [pH 7.5], 120 mM NaCl, 5 mM EDTA, and 0.5% NP-40 and 1 mM dithiothreitol, protease inhibitors as described above, and phosphatase inhibitors [50 mM NaF and 0.1 mM Na3VO4]. Lysates were immunoprecipi- tated with antibody to cyclin D1 (Upstate Biotechnology, Inc. [UBI]), and the immune complexes were collected on protein A-Sepharose beads (Santa Cruz Biotechnology), washed three times with lysis buffer, and boiled in SDS-gel loading buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotmed with anti-cdk4 antibodies. To analyze cyclin D1, cyclin E, or cdk4 in U343 cells was performed with whole-cell extracts made in NP-40 lysis buffer as described above. Lysates were separated by SDS-PAGE on a 12% gel. For immunoblot analysis of Id3 protein, lysates were prepared in RIPA buffer as described above and proteins were fractionated by SDS-PAGE on a 15% gel. Proteins were transferred to a nitrocellulose filter (Hybond-C Extra; Amersham), and the filter was blocked overnight in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) and 5% low-fat dried milk. Incubation with the appropriate primary antibody was done in PBS-T for 1 h at room temperature. Antibodies to Id3 (Santa Cruz Biotechnology), cyclin D1 (UBI), cyclin E (UBI), and cdk4 (PharMingen) were all used at 1:1,000 dilution. After three washes in PBS-T, the filters were incubated with horseradish peroxidase-conjugated immunoglobulin G (GIBCO) at a 1:10,000 dilution in PBS-T for 1 h and then washed extensively in PBS-T. The immune complexes were visualized with an enhanced chemiluminescence detect- ion system (Amersham). For G1 cyclin and cdk4 analysis of U343 transfectants, a single blot was probed sequentially with the antibodies to cyclin D1, cdk4, and cyclin E. Immunoblotting for p130 in U343, SF210, and U2OS cells was performed as previously described (34). For immunoprecipitation experiments, Saos-2 cells were transfected by the calcium phosphate method. Forty-eight hours after transfection, [35S] methionine-labeled cells were lysed by two cycles of freeze-thawing and suspended in immunoprecipitation buffer [50 mM N-2-hydroxyethylpiperazine-N’-2-ethane- sulfonic acid (HEPES), pH 7.5, 75 mM NaCl, 1 mM dithiothreitol, 0.5 μM bio- glycin-(p-aminomethyluter)-N,N,N’-tetraacetic acid (EGTA), 1 mM dithio- threitol, 0.1% Tween 20] containing 10% glycerol and protease inhibitors as indicated above. Immunoprecipitations were performed by incubating the lysates for 1 h at 4°C with rabbit antisera to Id1 (pMEP-p16) or with 1:100 affinity-purified rabbit antibody to p107 (C18; Santa Cruz Biotechnology), affinity-purified rabbit antibody to p130 (C20; Santa Cruz Biotechnology), or NRS. The antibodies against p107 and p130 used in these experiments did not show cross-reactivity against other members of the pRb protein family (data not shown). After incubation for 1 h at 4°C with protein A-Sepharose beads, immunoprecipitates were washed four times with immunoprecipitation buffer at 4°C. Immunopre- cipitated proteins were boiled in 2% SDS to dissociate complexes and diluted by the addition of RIPA buffer. Immunoprecipitations were performed as indi- cated with rabbit antisera to Id2. Immunocomplexes were finally collected on protein A-Sepharose beads, separated on an SDS–15% polyacrylamide gel, and visualized by autoradiography.

RESULTS

Id2 but not Id1 or Id3 binds pRb and pRb-related proteins. Id is a class of nuclear proteins characterized by structural and functional similarities (3, 4, 11, 19, 55, 62, 73). We previously showed that Id2, a member of the Id family, binds to pRb in vitro and in vivo (34). We therefore examined whether other members of the Id family could also interact with pRb. In vitro binding experiments were performed with fusion proteins consisting of GST and Id1 or Id3 and in vitro-translated pRb. As shown in Fig. 1A, neither GST-Id1 (lane 2) nor GST-Id3 (lane 3) bound to pRb under conditions that allowed a readily de- tectable binding of GST-Id2 to pRb (lane 3). However, when tested for the ability to interact with the basic HLH protein E12, all three GST-Id proteins bound to this transcription factor with comparable efficiencies (data not shown). Because the HLH region of Id2 protein is sufficient for binding to pRb (reference 34 and Fig. 1B, lane 4), we investigated whether the HLH domain of human Id1 and Id3 retained the ability to associate with pRb. Although the HLH domains of Id1, Id2, and Id3 share common sequences, GST fusion proteins con-
taining only the HLH region from Id1 (amino acids 65 to 106) or Id3 (amino acids 40 to 81) did not associate with pRb (Fig. 1B, lanes 3 and 5).

Mutational analyses have suggested that the pocket domain of pRb is the region to which Id2 binds (34). In this region, pRb and the related proteins p107 and p130 show extensive homology (21, 28, 41). To determine whether Id2 can also bind p107 and p130 and if different Id proteins preferentially interact with specific members of the pRb family, GST fusion proteins containing Id1, Id2, or Id3 were tested for binding to [35S]methionine-labeled, in vitro-translated lysates from Saos-2 cells transiently transfected with recombinant vector expressing Id2 (lane 1), p107 and p130 (lanes 2 and 3), and Id2 and p130 (lanes 4 and 5) were immunoprecipitated (IP) with antibody against Id2 (lane 1), p107 (lane 3), or p130 (lane 5) or with NRS (lanes 2 and 4). The immunocomplexes were boiled in 2% SDS and reimmunoprecipitated with antibody to Id2 as described in Materials and Methods.

We next sought to determine whether the interactions between Id2 and p107 and between Id2 and p130 observed in vitro could also be detected in vivo. Saos-2 cells were cotransfected with Id2 and p107 or p130, and 48 h after removal of DNA precipitates, cells were labeled with [35S]methionine and lysed. The resulting extracts were immunoprecipitated with a specific antibody to p107 or p130. After washing, the presence of Id2 in immunoprecipitates of p107 or p130 was evaluated by denaturation followed by reimmunoprecipitation with antibody to Id2 and analyzed by SDS-PAGE. Significant amounts of Id2 protein were discernible in p107 immunoprecipitates of Id2 in immunoprecipitates of p107 or p130 were evaluated by denaturation followed by reimmunoprecipitation with antibody to Id2 and analyzed by SDS-PAGE. Significant amounts of Id2 protein were discernible in p107 immunoprecipitates (Fig. 2D, lane 4). A less intense signal for Id2 was detected in p130 immunoprecipitates (Fig. 2D, lane 5). These results demonstrate that a specific interaction between Id2 and p107 or p130 occurs in vivo.

Specificity of Id2 as a functional antagonist of pRb, p107, and p130. Transfection of a pRb expression construct into sensitive cells leads to growth arrest in G1 (23, 32, 54). We showed previously that in addition to binding pRb, Id2 reverses the pRb-mediated cell cycle arrest when coexpressed with pRb in the human osteosarcoma cell line Saos-2 (34). To determine whether Id1 or Id3 overexpression could also interfere with pRb-induced cell cycle arrest, we evaluated BrdU incorporation as a measure of cell proliferation in transfected Saos-2 cells. A plasmid expressing the cell surface protein CD20 (72) was cotransfected to identify successfully transfected cells. The number of CD20-positive cells that were also positive for BrdU was determined 3 days after transfection by double immunofluorescence staining as described previously (34). As expected, Id2 completely reversed the growth inhibition induced by pRb expression (Table 1). However, cotransfection of the Id1 or Id3 expression plasmid with pRb resulted in the same
percentage of BrdU-positive cells as observed in cultures transfected with pRb alone (Table 1). Parallel immunofluorescence experiments were designed to verify the expression of transfected Id1, Id2, and Id3. We found that levels of expression of all of these genes were comparable and not detectably modified by coexpression of pRb. Also, expression of pRb was not affected by coexpression of any Id gene (data not shown).

Thus, although previous reports documented a virtually complete functional redundancy of Id proteins as inactivators of basic HLH transcription factors (49, 62), our results indicate that the reversal of growth suppression mediated by pRb is exclusively exerted by Id2.

Expression of p107 and p130 in Saos-2 cells efficiently inhibits cell cycle progression (66, 72). To evaluate the possibility of a functional interaction of Id2 with p107 and p130, we determined whether Id2 could rescue p107- and p130-induced growth suppression by using the strategy employed to show the functional interaction between Id2 and pRb. We cotransfected Id2 and p107 or p130 along with pCD20 into Saos-2 cells, and CD20-positive cells that were also positive for BrdU were scored. Consistent with its ability to bind p107 and p130, Id2 efficiently antagonized the cell cycle block caused by overexpression of p107 and p130 in Saos-2 cells (Table 1). Double immunofluorescence assays revealed that the expression of neither exogenous p107 and p130 nor Id2 was influenced by the presence of the cotransfected gene (data not shown). These results demonstrate that Id2 can functionally inactivate pocket proteins and suggest that the physical association between Id2 and pRb family members (Fig. 2) is required for the functional antagonism between these two classes of proteins.

Reversal of p16- and p21-induced cell cycle arrest by Id2 but not Id1 or Id3. Because pRb-induced inhibition of cell cycle progression was exclusively reversed by Id2, we speculated that the cellular growth-inhibitory factors acting upstream of pRb would be functionally inactive in cells constitutively expressing Id2 but not in cells overexpressing Id1 or Id3. CKIs (60) inhibit cyclin-cdk complex activity and act as growth inhibitors and tumor suppressors in many different cell types at least in part by preventing pRb phosphorylation (18, 26, 58). We therefore sought to determine whether overexpression of Id2 would render the cells refractory to the growth-inhibitory effect of CKIs p16 and p21. The growth-suppressive activities of CKIs p16 and p21 were examined in cells constitutively expressing Id1, Id2, or Id3 by a colony-forming efficiency assay (54). The human osteosarcoma cell line U2OS was stably transfected with an expression plasmid encoding Id1 (U2OS-Id1), Id2 (U2OS-Id2), or Id3 (U2OS-Id3) or with vector DNA alone (U2OS-vector). The expression of these exogenous genes was verified in pools of cells maintained in medium supplemented with G418 by immunoprecipitation of Id1 and Id2 (Fig. 3, lanes 2 and 4, respectively) or by Western blotting of Id3 (Fig. 3a, lane 6). Parallel plates containing the same number of U2OS-Id1, U2OS-Id2, U2OS-Id3, and U2OS-vector cells were transfected with one of three plasmid DNAs carrying the hygromycin resistance gene, pMEP4, pMEP-p16, or pMEP-p21, and the number of hygromycin-resistant colonies per plate was scored 14 days later. The results are expressed in Fig. 3b as the percentage of colony numbers obtained in cultures transfected with p16 or p21 compared with the numbers of colonies obtained in parallel cultures transfected with the empty vector. Forced expression of p16 or p21 in U2OS cells engineered to

### Table 1. Effects of Id proteins on cell cycle arrest mediated by pRb and pRb-related proteins in Saos-2 cells

| Transfected DNA | % of transfected cells in S phase
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<tr>
<td>p107 + Id2</td>
<td>17</td>
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* Saos-2 cells were cotransfected by the calcium phosphate method with pCMV-CD20 DNA together with the indicated plasmids (vector 1, pRbActin; vector 2, pCMV). Forty-eight hours after removal of the DNA precipitates, cells were incubated in medium containing BrdU for 2 h. Double immunofluorescence was used to determine the percentage of CD20-positive cells that were stained with anti-BrdU antibody. Samples from parallel transfections were immunostained to verify the expression of transfected genes (data not shown). Results of two independent experiments which are representative of multiple experiments performed are shown.
expression of Id proteins resulted in alterations of cyclins and/or cdks involved in G₁ control. Two human glioma cell lines were chosen for these experiments. The cell line U343 contained an equal ratio of hyperphosphorylated and underphosphorylated forms of pRb (Fig. 4A, lane 2), a pattern similar to that observed in U2OS cells (Fig. 4A, lane 1) and typical of cells expressing a wild-type copy of the RB gene (64). We could not detect any band corresponding to pRb in SF210 cells (Fig. 4A, lane 3). Pools of cells constitutively expressing either Id1 or Id2 were obtained from cell lines U343 (U343 CMVId1 or U343 CMVId2, respectively) and SF210 (SF210 CMVId1 or SF210 CMVId2, respectively) (Fig. 4B). Overexpression of Id2 stimulated the growth of U343 (pRb-positive) cells but did not affect the growth rate of SF210 (pRb-negative) cells (Fig. 4C). This finding is consistent with our previous observation that Id2 stimulates the growth of U2OS (pRb-positive) cells but lacks growth-stimulatory activity in Saos-2 (pRb-negative) cells (34). The expression of high levels of Id1 did not enhance cell proliferation in either of these two glioma cell lines (Fig. 4C), and neither Id1 nor Id3 had growth-stimulatory activity in U2OS cells (data not shown).

We next characterized the pattern of expression of G₁-phase regulators in U343 cells by immunoblot analysis. We found that exponentially growing pools of U343 CMVVector and U343 CMVId1 cells expressed comparable levels of cyclin D1 (Fig. 5A). In contrast, in U343 cells overexpressing Id2, the level of cyclin D1 protein was greatly reduced. However, cyclin E, another G₁ cyclin, and cdk4, the main partner of cyclin D1, were present at similar levels in all three cell lines (Fig. 5A), suggesting a specific effect of Id2 expression on the regulation of cyclin D1. We also evaluated the interaction of cyclin D1 with cdk4 by coupled immunoprecipitation-Western blotting experiments. Equal amounts of cellular protein from exponentially growing U343 CMVvector, U343 CMVId1, and U343 CMVId2 cells were immunoprecipitated with an antibody specific for cyclin D1, fractionated by SDS-PAGE, and evaluated by Western blotting with an anti-cdk4 antibody. As shown in Fig. 5A, cdk4 protein could not be coimmunoprecipitated by an anti-cyclin D1 antibody from U343 CMVId2 cells, although readily detectable amounts of this protein were present in immunoprecipitates from U343 CMVvector and U343 CMVId1 cells. In U343 CMVId2 cells, the absence of detectable cyclin D1-cdk4 complexes was not associated with an up-regulation of p16 protein (data not shown). To distinguish between a transcriptional and a posttranslational effect of Id2 on cyclin D1 levels, we examined the expression of cyclin D1 mRNA in U343 transfectedants (Fig. 5B). Consistent with the diminished expression of cyclin D1 protein, the level of cyclin D1-specific mRNA was lower in cells overexpressing Id2 than in cells overexpressing Id1 or transfected with vector DNA. Together, these data suggest that in cells overexpressing Id2, reduced expression of cyclin D1 mRNA is the likely event leading to decrease of cyclin D1 protein and consequent disappearance of cyclin D1-cdk4 complexes.

**DISCUSSION**

The role of Id proteins as inhibitors of terminal differentiation has been established for several different cell lineages (3, 13, 35, 38, 70). It has been proposed that, while preventing differentiation, expression of Id proteins is also responsible for keeping the cell in a highly proliferative state (1, 29, 34, 51). In this report, we address how Id protein expression affects the functions of key regulators of cell cycle progression. Our data indicate that only one of three Id proteins drives cell proliferation by antagonizing multiple factors that normally inhibit

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**TABLE 2. Effects of Id2 on p16- and p21-induced cell cycle arrest**

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* U2OS cells were cotransfected by the calcium phosphate method with pCMV-CD20 DNA together with the indicated plasmid (vector 1, pCMV; vector 2, pMEP4). Forty-eight hours after removal of DNA precipitates, cells were incubated in medium containing BrdU for 2 h. Double immunofluorescence was used to determine the percentage of CD20-positive cells that were stained with anti-BrdU antibody. Samples from parallel transfections were immunostained to verify the expression of transfected genes (data not shown). Results of two independent experiments which are representative of multiple experiments performed are shown.
progression from G₁ to S phase of the cell cycle. We found that overexpression of Id2 not only inactivated pRb but also abrogated the cell cycle-inhibitory activity of the pRb-related proteins p107 and p130. Conversely, in our experiments testing the effects of Id proteins on pRb-induced cell cycle arrest, neither Id1 nor Id3 had the ability to abolish the growth-suppressive activity of pRb. Also, neither Id1 nor Id3 interacted with pRb or pRb-related proteins. These results underscore the functional significance of the direct association between Id2 and pRb family members. In particular, the HLH region of Id2 mediates an exclusive association with the pocket domain of pRb (34) and likely with the homologous regions of p107 and p130, which is necessary (although it may not be sufficient) to antagonize the growth inhibition caused by proteins of the pRb family. Obviously, the very few nonconservative amino acid differences in the HLH of Id2 compared with the corresponding domains from Id1 and Id3 represent the most attractive candidates for conferring the specificity of binding to the pRb family members. To our knowledge, these results identify for the first time a distinct functional role for one of the Id family members.

The ability of Id2 to abolish the growth-suppressive effects of pRb, p107, and p130 suggests an intriguing explanation for the biphasic pattern of Id2 expression in early and late G₁ phase of the cell cycle following growth factor stimulation of arrested cells (1, 29). Id2 may be required for sequential inactivation of distinct targets represented by different members of the pRb family, which would otherwise restrain cell cycle progression through timely regulated associations with the transcription factor E2F. In this model, the first peak of Id2 expression functions to disrupt the association between E2F and p130, thus removing the block imposed on quiescent cells in G₀ (66). Later in G₁, the second peak of Id2 expression liberates E2F from the constraints derived from its associations with pRb and p107 (6, 12, 40, 61). Therefore, the physical interaction between Id2 and p130, pRb, and p107 would represent the mechanism for sequestering distinct pocket proteins at different times during G₁ to allow cell cycle progression (Fig. 6).

Proper control of pRb function is a central feature in regulation of cell cycle progression, and continuous cellular proliferation requires extinction of pRb kinase activity by CKIs. Conversely, overexpression of CKIs inhibits cell growth presumably as a result of the inhibition of pRb phosphorylation by cyclin-cdk complexes (59). We have previously proposed that when Id2 and pRb are coexpressed, the unphosphorylated, active form of pRb is functionally inactivated by the physical association with Id2 (34). Here we speculate that under conditions in which Id2 levels are increased (after growth factor stimulation of cells arrested in the G₀-G₁ phase of the cell cycle or perhaps in certain tumor cell types [73]), the excessive

FIG. 4. Effects of constitutive expression of Id1 or Id2 on cell proliferation of U343 and SF210 glioma cells. (A) Immunoblot analysis of endogenous pRb in U343 and SF210 glioma cell lines. The human osteosarcoma cell line U2OS was used as a positive control. The positions of hypophosphorylated and hyperphosphorylated forms of pRb are indicated on the left (pRb and ppRb, respectively). (B) Expression of exogenous Id proteins in U343 and SF210 cells. Pools of U343 and SF210 cells transfected with pCMV-Id1, pCMV-Id2, or the CMV expression vector without an insert were analyzed by immunoprecipitation of [35S]methionine-labeled lysates to evaluate the expression of exogenous genes. α-Id1 and α-Id2, antibodies to Id1 and Id2. (C) Representative growth curve of U343 and SF210 cells overexpressing Id1 or Id2. Identical numbers of U343 and SF210 transfectants were plated on day 1. On the indicated days, the total number of viable cells was determined by trypan blue dye exclusion. Each point represents the mean ± the standard deviation of triplicate samples.
amount of unphosphorylated pRb resulting from CKI activity would be ineffective in mediating the cell cycle block because it would be completely titrated by Id2 (Fig. 6). Inactivation of CKIs would also be expected to occur following expression of viral oncoproteins, which also bind and inactivate pRb (14, 48, 69). Indeed, it was recently shown that suppression of cellular transformation by p16 can be overcome by the E1A oncoprotein (58) and that expression of simian virus 40 large T antigen reverses growth inhibition by p16 (47). Our results demonstrate that overexpression of Id2 in U2OS cells abolished the growth-inhibitory effects of p16 and p21, which are members of two distinct classes of CKIs, whereas overexpression of Id1 or Id3 was ineffective. This finding is consistent with the physical association of pRb with Id2 but not Id1 or Id3. However, in U2OS cells, Id2 inhibition of p16-mediated cell cycle arrest was consistently more efficient than inhibition of the p21-mediated block (Fig. 3b and Table 2), p16 is a selective inhibitor of cdk4 and cdk6 (46, 59), whose known substrates are pRb and possibly the other members of the pRb family, which all are bound and functionally inactivated by Id2 (Fig. 2 and Table 1). p21 protein is a universal inhibitor of cdk activity (18, 25, 30, 71), which probably phosphorylates unknown substrates in addition to pRb family members. The different spectra of cdk targets for p16 and p21 may therefore account for the different efficiencies with which Id2 can reverse p16- or p21-imposed cell cycle arrest. We proposed that Id2 completely abolishes the growth-inhibitory effect of p16 through its inactivation of pRb, while the residual activity of p21 in inhibiting G1 progression in the presence of Id2 may rely exclusively on its ability to prevent phosphorylation of substrates other than pocket proteins, which are not affected by Id2 expression. Consequently, in the cell line Saos-2, which does not express functional pRb, p21 still induces cell cycle arrest, but this effect cannot be reversed by Id2.

In this report, we also document a novel, specific property of the Id2 protein, the suppression of cyclin D1 mRNA and protein expression. Moreover, in cells expressing high levels of Id2, cyclin D1-ckd4 complexes are no longer detected. While we cannot exclude the contribution of posttranslational events in decreasing the levels of cyclin D1 protein, the substantial reduction of cyclin D1 mRNA may well explain the consequent decrease of cyclin D1 protein and the absence of cyclin D1-cdk4 complexes. Id2 may negatively regulate cyclin D1 expression through the inhibition of DNA binding by an unknown basic HLH factor involved in the positive control of cyclin D1. However, the absence of E boxes in the cyclin D1 promoter (52) and the lack of a similar effect on cyclin D1 expression in cells overexpressing Id1 do not support this hypothesis. Interestingly, cyclin D1 expression is down-regulated in response to the viral oncoproteins E1A and large T antigen (43, 50) and to the nuclear oncogene myc (52). These factors have been shown to bind and inactivate members of the pRb family, and pRb binding is important for their transforming abilities (14, 56, 69). Furthermore, cyclin D1 and cyclin D1-ckd4 complexes are dispensable for G1 progression in cells carrying an inactive pRb (42). Thus, the negative regulation of cyclin D1 in cells overexpressing Id2 but not Id1 seems to be part of a cellular adaptive response to the inactivation of proteins of the pRb family, which renders cells independent of the rate-limiting steps for G1 progression controlled by cyclin D1-ckd4 kinase activity (Fig. 6). These findings suggest strongly that the stimulation of cell proliferation by Id2 is a direct result of its interaction with pRb and pRb-related proteins. This association leads to the complete inactivation (p16) or partial (p107, p130, and p21) functional inhibition of tumor suppressor protein activity. The overexpression of Id2 observed in some tumor cells (73) may therefore contribute to make cancer cells refractory to an entire pathway of growth-inhibitory proteins whose function in normal cells is to restrain cell cycle progression.

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REFERENCES

6. Hernandez, M. C. Personal communication.