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-cdk4 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 oncoproteins blocks differentiation and prevents the normal G_0 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). G_1 cyclins functioning as regulatory subunits of their partners cyclin-de-pendent kinases (cdks) phosphorylate pRb and inactivate the growth-inhibitory function of pRb (20, 32). Phosphorylation by G_1 cyclin complexes has recently been proposed as a mechanism controlling p107 activity as well (2). Molecules that inhibit the activity of cdks (CKIs) have recently been identified. These include members of the CIP/KIP family (p21, p27, and p57) (18, 30, 39, 45, 53, 65), which act as universal inhibitors of cdk activity, and a distinct family of 15- to 20-kDa proteins, the INK proteins, which specifically inhibit cyclin D-dependent cdk4 and cdk6 activity (8, 26, 27, 33, 59). By inactivating the negative regulators of pRb, CKIs positively regulate pRb function.

Id proteins are a family of helix-loop-helix (HLH) proteins (3, 4, 11, 19, 55, 62, 73) lacking a basic amino acid domain necessary to bind DNA (3, 35). They are thought to function in a dominant negative manner by sequestering ubiquitously expressed (e.g., E12, E47, and E2-2) or cell-type-restricted (e.g., Tal-1 and myoD) basic HLH transcription factors (3, 62, 67), thereby blocking the binding of dimerized basic HLH proteins to DNA. This results in inhibition of lineage-specific gene expression and differentiation (3, 13, 35, 38, 70). In proliferating myoblasts, Id1 levels are high, and Id1 is complexed with E2A proteins. Under conditions that activate muscle differentiation, Id1 protein levels decrease, allowing the formation of E12/E47-myoD heterodimers, which in turn activate myogenesis (35). Simultaneously, withdrawal of these cells from the cell cycle occurs. Consistent with their role as inhibitors of differentiation, expression of Id genes is high in undifferentiated cells and in tumor cells (19, 73).

Recently we reported that Id2 physically associates with the hypophosphorylated, active form of pRb, and we proposed that the interaction of Id2 with pRb results in a loss of pRb-mediated growth suppression (34). We show here that Id2 but not Id1 or Id3 is able to associate with pRb and with the pRb-related proteins p107 and p130. We also demonstrate that

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Id2 expression reverses p107- and p130-induced cell cycle arrest. Furthermore, in the presence of high levels of Id2, the CKIs p16 and p21 no longer exert their growth-suppressing activities and G_1 cyclin-cdk complexes undergo important alterations.

MATERIALS AND METHODS

Cell cultures, transfection, and growth curve. Human osteosarcoma cell lines Saos-2 and U2OS and human glioma cell lines U343 and SF210 were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Transfection of all cells was performed by the calcium phosphate method. pCMV-Id2 has been previously described (34). To generate expression plasmids pCMV-Id1 and pCMV-Id3, the full-length coding regions of human Id1 and Id3 were cloned into a cytomegalovirus (CMV)-based expression vector. Stable transformants from U2OS, U343, and SF210 cells were maintained in complete culture medium containing 600 μg of G418 per ml. All subsequent experiments involving the use of stable transfectants were performed in the presence of G418. To determine the growth rates of stable transfectants from U343 and SF210, cells were seeded in six-well plates in complete medium. Medium was changed every 3 days. Triplicate cultures were counted at daily intervals by hemocytometer, and cell viability was determined by trypan blue dye exclusion.

In vitro binding assay. [^{35}S]methionine-labeled proteins were prepared in vitro, using a reticulocyte lysate system (Promega). Plasmids for in vitro transcription of RNAs, pRb (37), p107 (72), p130 (41), and p300 (17), were described previously. Glutathione S-transferase (GST)–Id2, GST-δHLH Id2 (a GST-Id2 fusion protein in which the HLH region of Id2 has been deleted), and GST-HLH Id2 (a GST-Id2 fusion protein including only the HLH region of Id2) were described in a previous report (34). GST-Id1 and GST-Id3 (31a) included the full-length coding region of human Id1 (73) and mouse Id3 (11). To generate pGST-HLH Id1 and pGST-HLH Id3, the HLH regions of human Id1 (codons 65 to 106) and Id3 (codons 40 to 81) were obtained by PCR amplification and directionally cloned in the *Bam*HI and *Eco*RI sites of pGEX-2T (Pharmacia). Expression and purification of GST fusion proteins and in vitro binding experiments were performed as previously described (34).

S-phase analysis and colony-forming efficiency assay. S-phase evaluation of transfected 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 CD20 (Beckton Dickinson) and with a fluorescein-conjugated monoclonal antibody to BrdU (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, U2OS-Id2, and U2OS-Id3 cells (see 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 10^6 per dish. Samples (20 μg) of recombinant plasmid DNAs consisting of the expression vector pMEP4 (Invitrogen) carrying the hygromycin resistance gene and the full-length coding region of p16 or p21 (pMEP-p16 or pMEP-p21, respectively) were transfected into the cells by the calcium phosphate method. Forty-eight hours after transfection, cells were split 1:8 and 1:16 into medium containing G418 (600 μg/ml) and hygromycin (170 μg/ml). Fourteen days after selection in hygromycin, colonies with more than 32 cells were stained with crystal violet and counted under the microscope. Colonies were scored from entire plates, and the numbers of colonies in control cultures were found to vary from 80 to 130 per plate. The effect of p16 and p21 expression on growth was determined for each cell line by calculating the ratio between the number of hygromycin-resistant colonies in plates transfected with pMEP-p16 or pMEP-p21 and the number of colonies in plates transfected with the vector pMEP4 DNA. The mean values between the dish containing a 1:8 dilution and the dish containing a 1:16 dilution are reported.

Immunoprecipitation, Western blotting (immunoblotting), and coimmunoprecipitation. To immunoprecipitate Id1 and Id2, [^{35}S]methionine-labeled cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.7], 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 aprotinin 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 Biotechnology, Inc.), immunoprecipitates were washed four times in RIPA buffer, denatured 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 (10^7) were lysed in 1 ml of NP-40 buffer (50 mM Tris HCl [pH 7.6], 120 mM NaCl, 5 mM EDTA, and 0.5% NP-40 with 1 mM dithiothreitol, protease inhibitors as described above, and phosphatase

inhibitors [50 mM NaF and 0.1 mM Na$_3$VO$_4$]). Lysates were immunoprecipitated 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 immunoblotted with anti-cdk4 antibody (Pharmingen). Immunoblot analysis of 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 at 4°C 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 a 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 detection system (Amersham). For G_1 cyclins and cdk4 analysis of U343 transfectants, a single blot was probed sequentially with the antibodies to cyclin D1, cdk4, and cyclin E. Immunoblotting for pRb in U343, SF210, and U2OS cells was performed as previously described (34).

For coimmunoprecipitation experiments, Saos-2 cells were transfected by the calcium phosphate method. Forty-eight hours after transfection, [^{35}S]methionine-labeled cells were lysed by two cycles of freeze-thawing and suspended in immunoprecipitation buffer [50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM dithiothreitol, 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 antiserum to Id2 (1:50) or with 1 μg of 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 the 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. Immunoprecipitated proteins were boiled in 2% SDS to dissociate complexes and diluted by the addition of RIPA buffer. Reimmunoprecipitations were performed as indicated with rabbit antiserum to Id2. Immunocomplexes were finally collected on protein A-Sepharose beads, separated on an SDS–15% polyacrylamide gel, and visualized by autoradiography.

Northern (RNA) blot analysis. Total RNA was prepared from exponentially growing cells by extraction with RNAzol (Tel-Test) and ethanol precipitation. Samples of 20 μg were denatured and fractionated by electrophoresis in a 1.2% agarose-formaldehyde gel. After transfer to a nylon membrane (Schleicher & Schuell) in 10× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), the RNAs were hybridized to a ^{32}P-labeled random-primed cyclin D1 cDNA at 65°C for 24 h in 200 mM NaPO$_4$ (pH 7)–1 mM EDTA–15% formamide–5× Denhart's solution–7% SDS. The filter was washed at 65°C in 0.1× SSC–0.1% SDS and exposed to Kodak X-Omat film. The blot was stripped by boiling for 10 min in 0.5% SDS prior to reprobing with a glyceraldehyde-3-phosphate dehydrogenase probe.

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 5) bound to pRb under conditions that allowed a readily detectable 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-
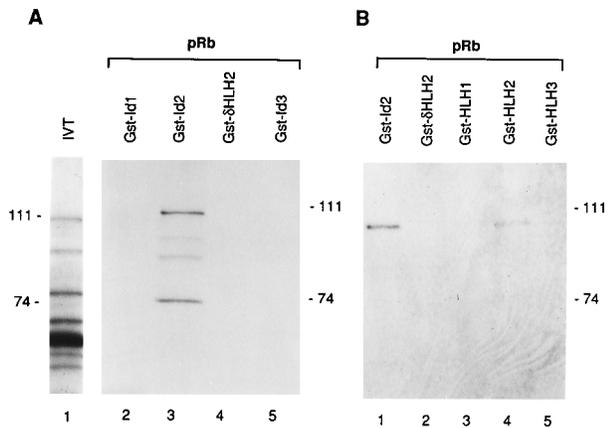
FIG. 1. Id2, but not Id1 or Id3, binds pRb. (A) [³⁵S]methionine-labeled pRb was synthesized in a rabbit reticulocyte lysate system (Promega). In vitro-translated [³⁵S]methionine-labeled pRb was incubated with Sepharose bead-immobilized GST-Id1 (lane 2), GST-Id2 (lane 3), GST-δHLH Id2 (lane 4), or GST-Id3 (lane 5). Bound proteins were eluted and resolved by SDS-PAGE. The labeled proteins were visualized by autoradiography. Lane 1 (in vitro translated [IVT]) contains 1/10 of the reaction product used in the binding experiment, which was run on a separate gel and therefore has a mobility slightly different from that in lane 3. (B) In vitro-translated [³⁵S]methionine-labeled pRb was incubated with Sepharose bead-immobilized fusion proteins consisting of GST and the HLH domain of Id1 (GST-HLH1; lane 3), Id2 (GST-HLH2; lane 4), or Id3 (GST-HLH3; lane 5). In lane 1, the binding of full-length GST-Id2 to pRb is shown. GST-δHLH Id2 (lane 2) was used as a negative control. Molecular size markers in kilodaltons are indicated.

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 the ability to bind [³⁵S]methionine-labeled, in vitro-translated p107 and p130. Figures 2A and B show that GST-Id2 could bind both p107 and p130, while GST-Id1 and GST-Id3 fusion proteins were unable to associate with these pRb-related proteins. Consistent with our previous observation that the HLH region of Id2 is necessary for pRb binding (reference 34 and Fig. 1), deletion of the HLH domain of Id2 in the construct GST-δHLH Id2 abolished the binding to p107 and p130 (Fig. 2A and B). Together, these results demonstrate that binding to pRb and pRb family members is an exclusive property of Id2 and suggest that this specificity is determined by the very few differences in the amino acid residues present in the HLH region of Id2 compared with the HLH regions of Id1 and Id3.

We also evaluated whether Id1, Id2, or Id3 could bind to p300, a cellular protein which interacts with the viral oncoprotein E1A but lacks the pocket domain conserved throughout the pRb protein family (17). As shown in Fig. 2C, all three GST-Id fusion proteins failed to interact with [³⁵S]methionine-labeled p300.

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
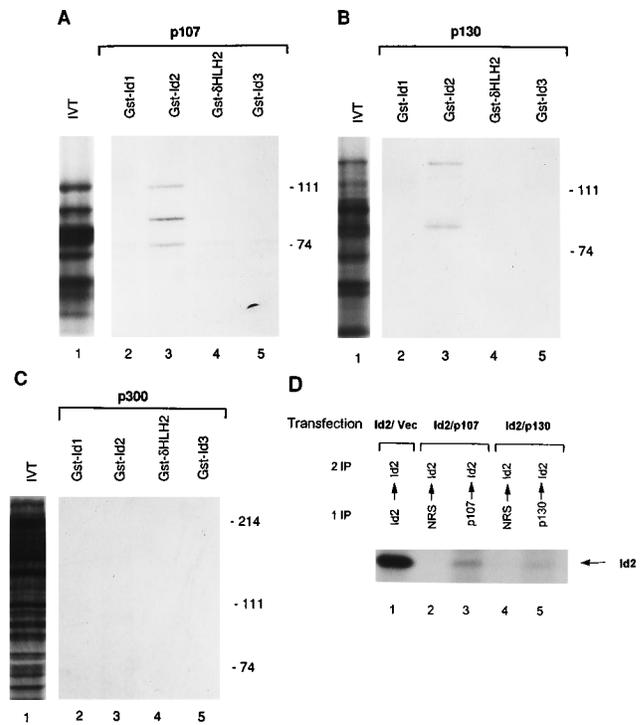

FIG. 2. Id2, but not Id1 or Id3, binds the pRb-related proteins p107 and p130. GST-Id1 (lane 2), GST-Id2 (lane 3), GST-δHLH Id2 (lane 4), and GST-Id3 (lane 5) were tested for binding to in vitro-translated [³⁵S]methionine-labeled p107 (A), p130 (B), or p300 (C) and analyzed as described for Fig. 1. In each panel, lane 1 (in vitro translated [IVT]) contains 1/10 of the translation reaction product used in the binding experiment. Molecular size markers in kilodaltons are indicated. (D) In vivo interaction between Id2 and p107 or p130. [³⁵S]methionine-labeled lysates from Saos-2 cells transiently transfected with recombinant vectors expressing Id2 (lane 1), Id2 and p107 (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.

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 (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

TABLE 1. Effects of Id proteins on cell cycle arrest mediated by pRb and pRb-related proteins in Saos-2 cells[a]

Transfected DNA	% of transfected cells in S phase	
	Expt 1	Expt 2
Vector 1 + vector 2	26	24
Vector 1 + Id1	23	24
Vector 1 + Id2	24	23
Vector 1 + Id3	23	21
Rb + vector 2	6	7
Rb + Id1	6	9
Rb + Id2	26	24
Rb + Id3	5	10
p107 + vector 2	5	6
p107 + Id2	15	16
p130 + vector 2	7	6
p130 + Id2	17	17

[a] Saos-2 cells were cotransfected by the calcium phosphate method with pCMV-CD20 DNA together with the indicated plasmids (vector 1, pβActin; 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.

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
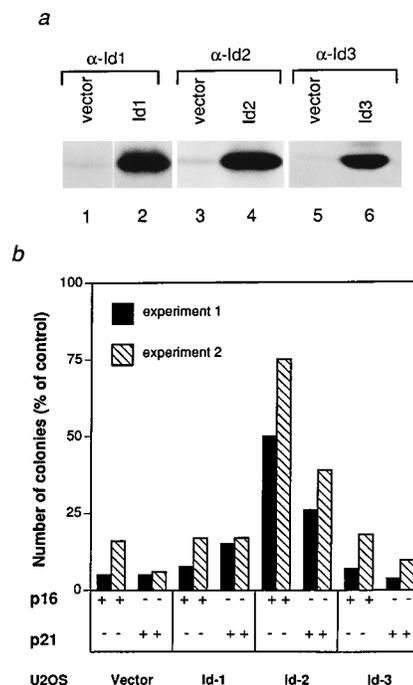

FIG. 3. Constitutive expression of Id2 prevents growth inhibition induced by p16 and p21 in U2OS cells. (a) Expression of Id proteins in U2OS transfectants. Cells were transfected with pCMV-Id1 (Id1), pCMV-Id2 (Id2), and pCMV-Id3 (Id3) (lanes 2, 4, and 6, respectively) or with the CMV expression vector without an insert (vector; lanes 1, 3, and 5). Pools of G418-resistant clones from each transfection were examined for constitutive expression of Id proteins. Id1 and Id2 were evaluated by immunoprecipitation of [35S]methionine-labeled cells (lanes 1 to 4), and immunoblot analysis was performed to detect Id3 (lanes 5 and 6). α-Id1, α-Id2, and α-Id3, antibodies to Id1, Id2, and Id3. (b) A CKI p16 or p21 expression plasmid (pMEP-p16 or pMEP-p21, respectively) or vector pMEP4 was transfected into U2OS cells stably transfected with vector pCMV alone or constitutively expressing high levels of Id1, Id2, or Id3 (see panel a). After transfection, the expression of the exogenous CKI genes was monitored by cellular immunostaining and found to be indistinguishable among the various transfectants (data not shown). Hygromycin-resistant colonies were stained and counted after 14 days of selection. The results are expressed as the percentage of colony numbers obtained in cultures transfected with p16 or p21 compared with the numbers of colonies obtained in cultures transfected with the empty vector. Results are shown for two independent experiments (hatched and filled bars).

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. 3a, 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 2. Effects of Id2 on p16- and p21-induced cell cycle arrest[a]

Transfected DNA	% of transfected cells in S phase			
	U2OS		Saos-2	
	Expt 1	Expt 2	Expt 1	Expt 2
Vector 1 + vector 2	25	24	21	21
Id2 + vector 2	27	26	21	23
Vector 1 + p16	7	7		
Vector 1 + p21	10	9	9	8
Id2 + p16	25	25		
Id2 + p21	17	15	11	12

[a] 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.

express high levels of Id1 or Id3 or stably transfected with the vector DNA led to a similar, marked decrease in the number of colonies (Fig. 3b). In contrast, the inhibition of colony formation by p16 overexpression was greatly diminished in U2OS cells overexpressing Id2 (Fig. 3b). Inhibition by p21 was also diminished, albeit to a lesser degree, in U2OS-Id2 cells (Fig. 3b). These results indicate that Id2 can relieve the growth suppression imposed by p16 and p21 and that Id1 and Id3 lack this function.

Ability of Id2 to relieve CKI-induced cell cycle arrest correlates with the presence of a functional pRb. Overexpression of p16 prevents proliferation in pRb-positive cells but is ineffective in pRb-negative cells (26, 36, 44, 47, 58). To examine the ability of Id2 to relieve cell cycle arrest by p16, we evaluated DNA synthesis in U2OS cells cotransfected with p16 and Id2 by immunostaining of BrdU. The surface marker CD20 was used to identify transfected cells as in Table 1. Consistent with results recently published by others (36, 44, 47), transient expression of p16 significantly decreased the number of BrdU-positive cells (Table 2). In contrast, the cell cycle-inhibitory effect of p16 was completely abolished in the presence of high levels of Id2 (Table 2). Unlike p16-mediated growth arrest, p21-mediated growth arrest does not absolutely require pRb (30, 58). To investigate the antagonistic effect of Id2 on the p21-mediated cell cycle block and to determine whether this effect requires pRb, transient transfection experiments were performed with U2OS (pRb-positive) and Saos-2 (pRb-negative) cells. Cells were cotransfected with p21 and Id2, and the percentage of transfected cells in S phase was evaluated by double immunostaining for BrdU and CD20. Overexpression of p21 caused similar levels of inhibition of entry into S phase in both U2OS and Saos-2 cells. However, coexpression of Id2 substantially decreased the cell cycle block induced by p21 in U2OS cells but was only minimally effective in Saos-2 cells (Table 2). Coexpression of exogenous Id2 and p16 or of Id2 and p21 was demonstrated by double immunofluorescence in parallel transfections (data not shown). These results are consistent with a model in which Id2-mediated reversal of the cell cycle block by two different classes of CKIs requires the presence of functional pRb.

Id2 affects expression of cyclin D1, leading to loss of cyclin D1-cdk4 complexes. Expression of Id proteins has been implicated in progression of cells through the G_1 phase of the cell cycle (1, 29, 34). We sought to determine whether exogenous

expression of Id proteins resulted in alterations of cyclins and/or cdks involved in G_1 control. Two human glioma cell lines were chosen for these experiments. The cell line U343 contained an equal ratio of hyperphosphorylated and under-phosphorylated 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 G_1-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 G_1 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 transfectants (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
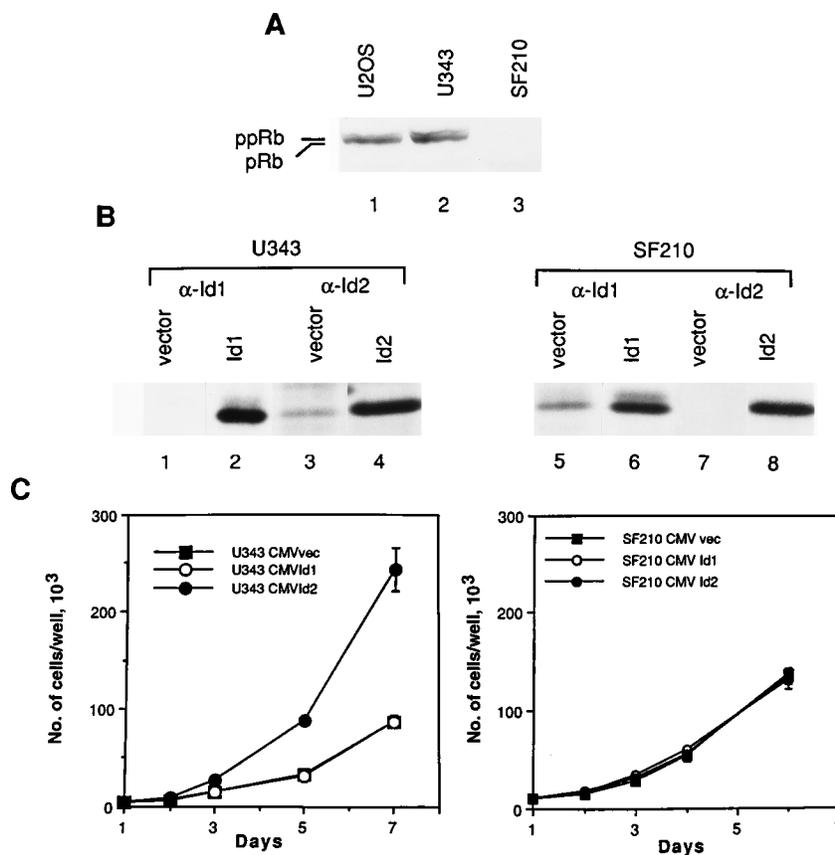
FIG. 4. Effects of constitutive expression of Id1 or Id2 on cell proliferation of U343 and SF210 glioma cells. (A) Immunoblot analysis of endogenous pRb in U343 and SF210 glioma cell lines. The human osteosarcoma cell line U2OS was used as a positive control. The positions of hypophosphorylated and hyperphosphorylated 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 [^{35}S]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.

progression from G_1 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 G_1 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 G_0 (66). Later in G_1, 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 G_1 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 G_0-G_1 phase of the cell cycle or perhaps in certain tumor cell types [73]), the excessive
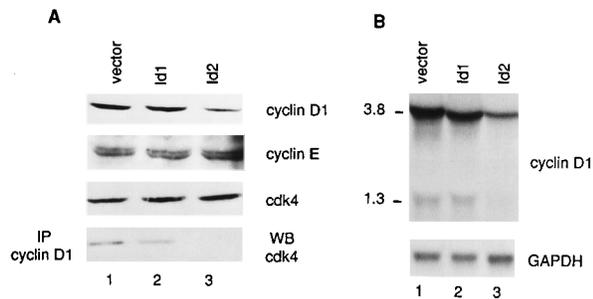
FIG. 5. Effects of Id2 expression on cyclin D1 and cyclin D1-cdk4 association. (A) Western blot (WB) and immunoprecipitation (IP) analysis of G_1 cyclins and cdk4 and cyclin D1-cdk4 complexes in U343 transfectants. Proteins from cell lysates of exponentially growing U343 CMVvector (lane 1), U343 CMVId1 (lane 2), and U343 CMVId2 (lane 3) cells were resolved by SDS-PAGE and probed with antibodies to cyclin D1, cyclin E, and cdk4. To evaluate cyclin D1-cdk4 association, cyclin D1 immunoprecipitates were probed with antibodies to cdk4 (lowest panel). (B) Northern blot analysis of cyclin D1 transcripts in U343 transfectants. The loading in each lane was monitored by the signal for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

FIG. 6. S-phase-promoting events of Id2 expression within the G_1 control pathway. Progression from G_0 to G_1 is stimulated by inactivation of hypophosphorylated pRb and possibly p107 and p130 by Id2. Enhanced Id2 expression which inactivates pRb is associated with an inhibition of cyclin D expression.

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 G_1 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 expres-
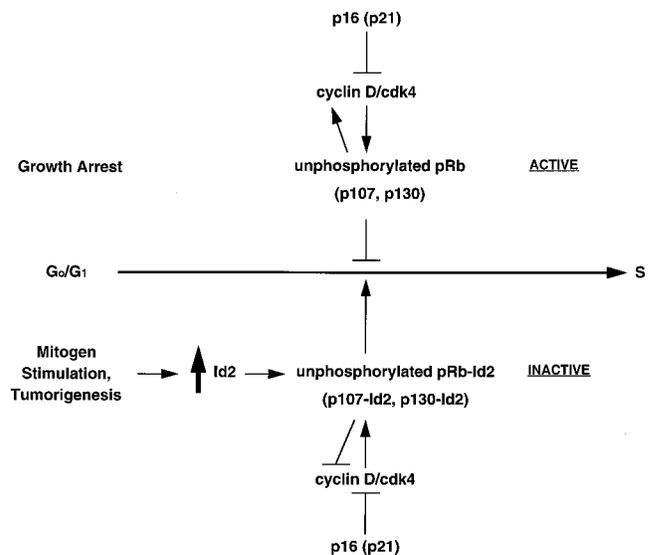
sion through the inhibition of DNA binding by an unknown basic HLH factor involved in the positive control of cyclin D1. However, the absence of E boxes in the cyclin D1 promoter (52) and the lack of a similar effect on cyclin D1 expression in cells overexpressing Id1 do not support this hypothesis. 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-cdk4 complexes are dispensable for G_1 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 G_1 progression controlled by cyclin D1-cdk4 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 (pRb and 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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