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Regulation of Id3 Cell Cycle Function by Cdk-2-Dependent Phosphorylation

RICHARD W. DEED,[1] EIJI HARA,[2]† GRAHAM T. ATHERTON,[1] GORDON PETERS,[2] AND JOHN D. NORTON[1]*

CRC Department of Gene Regulation, Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Manchester M20 9BX,[1] and Imperial Cancer Research Fund Laboratories, London WC2A 3PX,[2] United Kingdom

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The functions of basic helix-loop-helix (bHLH) transcription factors in activating differentiation-linked gene expression and in inducing G_1 cell cycle arrest are negatively regulated by members of the Id family of HLH proteins. These bHLH antagonists are induced during a mitogenic signalling response, and they function by sequestering their bHLH targets in inactive heterodimers that are unable to bind to specific gene regulatory (E box) sequences. Recently, cyclin E–Cdk2- and cyclin A–Cdk2-dependent phosphorylation of a single conserved serine residue (Ser5) in Id2 has been shown to occur during late G_1-to-S phase transition of the cell cycle, and this neutralizes the function of Id2 in abrogating E-box-dependent bHLH homo- or heterodimer complex formation in vitro (E. Hara, M. Hall, and G. Peters, EMBO J. 16:332–342, 1997). We now show that an analogous cell-cycle-regulated phosphorylation of Id3 alters the specificity of Id3 for abrogating both E-box-dependent bHLH homo- or heterodimer complex formation in vitro and E-box-dependent reporter gene function in vivo. Furthermore, compared with wild-type Id3, an Id3 Asp5 mutant (mimicking phosphorylation) is unable to promote cell cycle S phase entry in transfected fibroblasts, whereas an Id3 Ala5 mutant (ablating phosphorylation) displays an activity significantly greater than that of wild-type Id3 protein. Cdk2-dependent phosphorylation therefore provides a switch during late G_1-to-S phase that both nullifies an early G_1 cell cycle regulatory function of Id3 and modulates its target bHLH specificity. These data also demonstrate that the ability of Id3 to promote cell cycle S phase entry is not simply a function of its ability to modulate bHLH heterodimer-dependent gene expression and establish a biologically important mechanism through which Cdk2 and Id-bHLH functions are integrated in the coordination of cell proliferation and differentiation.

Transcription factors characterized by the basic helix-loop-helix (bHLH) domain play an essential role in the regulation of cell growth and differentiation (24, 30). DNA binding of bHLH proteins to a consensus E box recognition sequence (CANNTG) is facilitated by the basic region located immediately N-terminal to the two amphipathic alpha helices encompassing the loop region, which together comprise the highly conserved HLH domain (24, 34). The HLH domain itself mediates dimerization, which is essential for DNA binding and transcriptional activation of target genes that are typically involved in driving cell lineage commitment and terminal differentiation (24, 30, 34). There are two distinct classes of bHLH proteins (18, 24). Class A bHLH proteins, exemplified by the E2A-encoded E12, E47, and E2-5/ITF proteins (14, 31), often referred to as the "E" proteins, are ubiquitously expressed, whereas the class B subset represents a rather larger collection of more-tissue-specific transcription factors such as MyoD (and its myogenic relatives) in muscle cells (32, 36, 42), TAL1/LYL-1 in hemopoietic cells (35), and NeuroD in neuronal cells (23). Although homodimers of class A bHLH proteins are known to be functional, most notably in the B-lymphocyte lineage (2, 3, 44), the more usual configuration for E box binding and transcriptional activation is a class A-class B bHLH heterodimer.

The activities of bHLH proteins are regulated through a variety of mechanisms, one of which involves heterodimerization with members of the Id family of HLH proteins (6). This distinct class of HLH protein, of which there are four members in mammals (Id1 to Id4) (24), is characterized by the absence of a basic DNA-binding domain. As a consequence, Id proteins are able to antagonize the DNA-binding and transcriptional activation functions of bHLH transcription factors through heterodimerization (6, 24), a process which also results in destabilization of the bHLH protein partner (8). In addition, enforced ectopic expression of Id genes in a variety of cell lineages can promote cell growth and inhibit differentiation, and this is typically manifested in a delayed exit from the cell cycle under differentiation-inducing conditions (1, 10, 17, 37, 41). These functional properties of Id proteins exactly mirror those of their bHLH counterparts, which, in addition to driving differentiation-linked gene expression, also function to inhibit cell proliferation, principally through an arrest in the G_1 phase of the cell cycle (7, 33, 40). Significantly, Id gene and protein expression is markedly up-regulated during the G_1 phase transition in response to mitogenic stimulation (4, 9, 13, 29). Furthermore, antisense oligonucleotide blockade and antibody microinjection experiments have suggested a requirement of Id function for entry of cells into S phase (4, 13, 33). A widely held view is that the opposing activities of bHLH and Id proteins represent a major control mechanism for determining cell fate.

Recently, a potential link has been established between the activities of Id-bHLH proteins and cell cycle regulation by the demonstration that the Id2 protein is a substrate for cyclin E-

* Corresponding author. Mailing address: CRC Department of Gene Regulation, Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Wilmslow Rd., Manchester M20 9BX, United Kingdom. Phone: (44) 161 446 3129. Fax: (44) 161 446 3109. E-mail: grgjdn@picr.cr.man.ac.uk.

† Present address: Department of Preventive Medicine, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokohi, Kamigyo-ku, Kyoto 602, Japan.

and cyclin A-dependent Cdk2 activity (12). Cdk2-dependent phosphorylation of a serine residue at position 5 in Id2 abolishes its ability to antagonize the formation of a model E12–MyoD–E-box-binding complex in in vitro band shift assays. Moreover, following mitogenic stimulation in vivo, phosphorylation of Id2 at serine 5 occurs during late G_1, at a time which correlates both with activation of cyclin E-Cdk2 and with the formation of a specific E box DNA-binding complex in vivo that can be abrogated with an excess of unphosphorylated Id2 protein (12). These observations suggested that Cdk2-dependent phosphorylation of Id proteins might alter their target specificities and that this might represent an important mechanism for modulating Id-bHLH functions in cell cycle control. We have now confirmed both of these predictions with respect to Id3, the only other well-characterized Id family member that possesses the conserved consensus Cdk phosphorylation site at serine 5. Like Id2, the Id3 protein becomes phosphorylated in late G_1, at a time coincident with the activation of cyclin A- and cyclin E-dependent Cdk2. In vitro phosphorylation of Id3 by Cdk2 abolishes its ability to antagonize E12 homodimer binding to an E box sequence. However, in contrast to Id2, phosphorylation of Id3 greatly enhances its ability to abrogate E12-MyoD-E box complex formation in vitro, and substitution of a highly charged aspartate residue at serine 5 (mimicking phosphorylation) similarly enhances its ability to inhibit E12-MyoD transactivation of an E-box-dependent reporter gene in vivo. However, compared with the wild-type protein, Asp5 Id3 is completely devoid of activity in promoting S phase of the cell cycle in transfected fibroblasts, whereas an Ala5 mutant Id3 (ablating phosphorylation) displays an activity significantly greater than that of the wild-type protein. Cdk2-dependent phosphorylation of Id3 during late G_1 therefore nullifies a cell cycle regulatory function of this Id protein which is known to be required during early G_1. Our findings also demonstrate that by analogy with their bHLH counterparts (7, 33, 40), the cell cycle regulatory functions of Id proteins in promoting S phase may be dissociable from their ability to modulate bHLH heterodimer-dependent gene expression.

MATERIALS AND METHODS

Purification of bacterially expressed Id proteins. The human Id1, Id2 (13), and Id3 (9) coding sequences and their various mutants were cloned into the pGEX-2T vector (Pharmacia) or the pRSET-B vector (Invitrogen) for expression in bacteria. The recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) pLysS. Glutathione *S*-transferase (GST)-Id proteins were purified according to published protocols (39). Histidine-tagged Id proteins produced in the pRSET-B vector were purified on nickel-charged chelating agarose as recommended by the supplier (Invitrogen).

In vitro kinase assays. In vitro kinase assays were performed essentially as described previously (12). Briefly, Sf9 insect cells were coinfected with the appropriate recombinant baculoviruses, and whole-cell extracts were prepared 48 h postinfection as previously described (20). Phosphorylation reactions (final volume, 40 μl) were performed in a solution containing 10 mM HEPES (pH 7.8), 1 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM ATP, and 5 μCi of [γ-^{32}P]ATP and incubated with 1 μg of GST-Id1, GST-Id2, or GST-Id3 bound to glutathione-Sepharose beads. After 15 min at 37°C, the reactions were terminated by the addition of 1 ml of an ice-cold buffer, NETN, containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40. The glutathione-Sepharose beads were recovered by centrifugation, washed several times in NETN, boiled, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 12% polyacrylamide gel. In some experiments, the Sf9 cell lysates were preincubated for 10 min at 30°C with 20 ng of histidine-tagged p21 protein.

Phosphate labelling and immunoprecipitation. Primary human B cells were isolated as described previously (29) and were rested overnight in medium (RPMI supplemented with 10% fetal calf serum [FCS]). Phorbol ester (PMA) was then added to 30 nM, and cells were labelled with 0.5 mCi of [^{32}P]orthophosphate per ml for 2 h prior to harvest at various times poststimulation. Parallel cultures were stimulated with PMA and labelled with [^{35}S]methionine as described previously (8). Labelled cells were washed in ice-cold phosphate-buffered saline (PBS), and Id3 protein was immunoprecipitated from radioimmunoprecipitation assay lysates (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate) with RD-1 polyclonal anti-Id3 antibody for analysis on SDS–12% polyacrylamide gels, as described elsewhere (8). For some experiments, labelled lysates were similarly prepared from transiently transfected COS cells and used for immunoprecipitation of Id3. Expression constructs (8) encoding wild-type Id3 and Id3 Ala5 and Asp5 mutants were in the pcDNA3 expression vector (Invitrogen), as were expression constructs encoding cyclin E, CdK2, p16, and p21.

Electrophoretic mobility shift assays. Unlabelled proteins were synthesized by coupled transcription and translation of plasmid DNA by using the TNT expression system (Promega) with either T7 or SP6 RNA polymerase. Samples (5 μl) of the different translation reaction mixtures were mixed and used for DNA-binding assays. An E box consensus oligonucleotide based on the muscle creatine kinase gene enhancer was used in all band shift assays (12). This was ^{32}P labelled with the Klenow fragment of *E. coli* DNA polymerase I and was used in conjunction with a mutant unlabelled oligonucleotide competitor in some experiments, as described previously (12).

DNA-binding reactions were done in a total volume of 20 μl comprising 20 mM HEPES (pH 7), 500 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 1 μg of double-stranded poly(dI-dC), and 0.2 ng of labelled double-stranded probe. The mixtures were preincubated without the labelled probe at room temperature for 10 min and for 15 min further at room temperature after addition of the labelled probe. The concentrations and purities of bacterially expressed (histidine-tagged) Id proteins used in various experiments were verified by PAGE using Coomassie blue staining. The binding reactions were then subjected to electrophoresis on a 6% polyacrylamide gel in 25 mM Tris (pH 8.3)–25 mM boric acid–0.5 mM EDTA at 150 V for 2 h at room temperature. The gels were dried, and labelled complexes were detected by autoradiography.

Analysis of gene regulatory and cell cycle functions of Id3. Mouse NIH 3T3 fibroblasts were transiently transfected by the calcium phosphate procedure with an E-box-driven chloramphenicol acetylase (CAT) reporter cassette together with E12 and MyoD expression vectors, as described previously (1). The reporter was challenged with increasing inputs of either wild-type or mutant Id expression constructs, and all transfections were normalized for efficiency by inclusion of a luciferase reporter. Quantitation of CAT and luciferase activity in cells at 24 h posttransfection was performed as described previously (1).

The two-hybrid luciferase assay was carried out essentially as described previously (8), except that a dual luciferase reporter system (Stop and Glo Luciferase; Promega) was used to facilitate monitoring of transfection efficiency. The plasmids encoding Vp16MyoD and Gal4E12 were constructed by insertion of mouse MyoD and human E12 cDNAs, modified by the addition of a *Bam*HI recognition site at the ATG start site, into *Bam*HI/*Eco*RI-digested pVp16 *Nco*I and *Psg* 424 vectors, respectively (8).

For analysis of Id3 effects on cell proliferation, primary rat embryo fibroblasts (cultured in Dulbecco modified Eagle medium supplemented with 10% FCS) were transiently transfected as above. The LacZ-expressing vector pCMVβGal (1) was included as a marker to permit immunofluorescent detection of transfected cells. Cells were pulse labelled with 10 nM bromodeoxyuridine (BrdU) for 4 h and then washed in PBS and fixed with 4% paraformaldehyde. After permeabilization in 0.2% Triton X-100 (15 min), cells were incubated in a 1/500 dilution of anti-LacZ antibody (5 Prime→3 Prime, Boulder, Colo.) overnight at 4°C, washed, and then incubated with a 1/100 dilution of biotin-labelled anti-rabbit antibody (Dako) at room temperature for 1 h. A 1/40 dilution of streptavidin-fluorescein isothiocyanate (Dako) was used for immunofluorescent detection of LacZ-expressing cells. For detection of incorporated BrdU, cells were then incubated with 2 M HCl for 20 min, washed in PBS, and then incubated with a 1/10 dilution of monoclonal antibody to BrdU for 30 min, essentially as described by the supplier (Boehringer). After a wash in PBS, a direct Texas red conjugate of secondary anti-mouse antibody was used at a 1/40 dilution. The expression of transfected Id protein was monitored by immunofluorescent staining in these experiments, essentially as described previously (8).

RESULTS

Cdk2-dependent phosphorylation of Id3 in vitro. To determine whether Id3 could be phosphorylated in vitro by cyclin-Cdk2 complexes, bacterially expressed Id proteins were incubated with cell extracts from baculovirus-infected Sf9 cells expressing combinations of cyclins and Cdk's. As shown in Fig. 1A, the Id1 protein, which lacks a consensus Cdk site, was not detectably phosphorylated by any of the combinations of cyclins or Cdk's used. Consistent with previous data (12), Id2 was phosphorylatable by cyclin A- and E-Cdk2 but not by cyclin D-Cdk4, and the same profile was displayed by the Id3 protein (Fig. 1A). Interestingly, cyclin A-Cdk2 appeared to phosphorylate Id3 more efficiently than did cyclin E-Cdk2 in the in vitro assays (compare the profiles of Id2 and Id3 in Fig. 1A), raising the possibility of substrate specificity of these Cdk2 complexes for Id3. Cyclin A- and E-Cdk2 phosphorylation of Id3 was

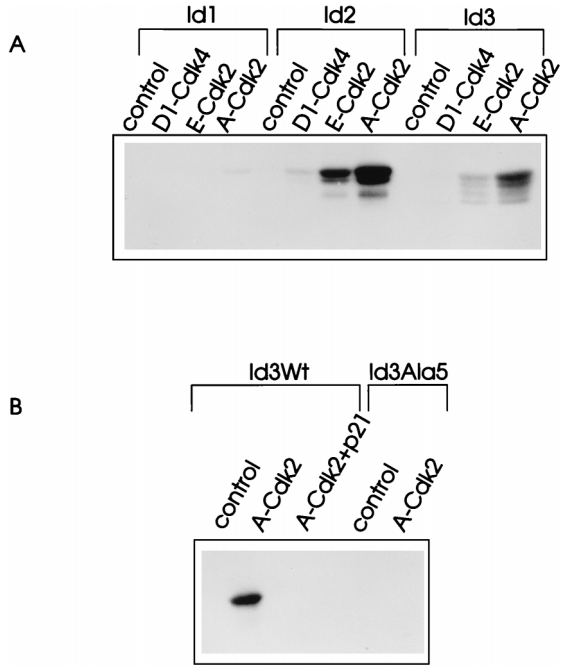

FIG. 1. In vitro phosphorylation of Id proteins by cyclin-Cdk2 complexes. (A) Sf9 insect cells were coinfected with baculovirus vectors encoding the appropriate cyclins and Cdk's in order to assemble functional complexes in vivo. Lysates were then incubated with the relevant GST-Id protein together with [γ-^{32}P]ATP. Control lysates were from uninfected Sf9 cells. The resulting labelled proteins were analyzed by SDS-PAGE with a 12% acrylamide gel. (B) A histidine-tagged Id3 protein, either the wild type (Wt) or having a mutation at serine 5 (Ser5-Ala5), was used for the in vitro kinase assay in the presence of the Cdk2 inhibitor, p21 (expressed as a His-tagged fusion protein).

completely abolished by the Cdk inhibitor, p21, and also by site-directed mutagenesis of Id3 at serine 5 (Ser5-Ala5), as shown in Fig. 1B.

Cdk2-dependent phosphorylation of Id3 in vivo. To investigate whether phosphorylation of Id3 occurs in vivo, primary human B cells were used, since this cell type expresses particularly abundant levels of Id3 protein. Following mitogenic stimulation, a transient peak of Id3 protein was detected by [^{35}S]methionine labelling at around 4 h poststimulation (Fig. 2A). This had declined by 16 h and was upregulated at 24 h, around the time of transition from G_1 to S phase of the cell cycle. By contrast, ^{32}P-labelled Id3 was immunoprecipitable only at these later time points (Fig. 2A), which coincided with the appearance of cyclin A- and E-Cdk2 activity (data not shown). The cell cycle kinetics of Id3 phosphorylation therefore appear to be identical to those previously reported for Id2 (12). Moreover, when expression constructs encoding either wild-type Id3 or the Ala5 mutant of Id3 were transfected into COS cells, only the wild-type protein was ^{32}P labelled, whereas both mutant and wild-type proteins were [^{35}S]methionine labelled (Fig. 2B). Phosphorylation was specifically abrogated by the Cdk inhibitor, p21 (Fig. 2C), but not by p16, which specifically targets Cdk4 and Cdk6. Essentially all of the in vivo-phosphorylated Id3 can therefore be accounted for by cyclin E- and A-Cdk2 phosphorylation of the serine 5 residue.

Cdk2-dependent phosphorylation of Id3 affects bHLH antagonism in vitro. Previously, phosphorylation of Id2 by cyclin A- and E-Cdk2 was shown to completely abrogate its ability to antagonize bHLH protein binding to a consensus E box oligonucleotide sequence in a model in vitro band shift system (12). We therefore used the same assay system to evaluate the functional consequences of phosphorylation of Id3. The Id1 protein, which is not detectably phosphorylated by cyclin A- or E-Cdk2, provided a convenient control in these experiments. As shown in Fig. 3A, specific complexes representing either an E12 homodimer or an E12-MyoD heterodimer were formed following incubation of in vitro-translated protein with a labelled E box oligonucleotide. Increasing the inputs of each of the three Id proteins (in the unphosphorylated state) gave qualitatively and quantitatively distinct profiles of inhibition of homodimer and heterodimer binding to DNA (Fig. 3B). Incubation of Id1 with cyclin-Cdk2 did not affect this inhibition, whereas in vitro phosphorylation of Id2 completely neutralized its function, as expected (Fig. 3C). However, cyclin A-Cdk2 phosphorylation of Id3 altered its bHLH-antagonizing properties. In contrast to Id3 protein in its unphosphorylated state, which inhibits E12 homodimer but not E12-MyoD heterodimer binding, the in vitro-phosphorylated Id3 protein inhibits exclusively the E12-MyoD heterodimer but not the E12 homodimer (Fig. 3C). Indeed, the intensity of the E12 homodimer complex was actually increased following addition of phosphorylated Id3 protein, presumably as a result of sequestration of the MyoD partner by phosphorylated Id3. Since MyoD does not form an E-box-binding homodimer (reference 22 and data not shown), we could not directly test this in band shift assays. As shown in Fig. 4, the Ala5 Id3 mutant behaved in a way similar to the wild-type protein. However, substitution of a highly charged aspartate residue at position 5 (Id3 Asp5) faithfully mimicked the properties of the in vitro-phosphorylated

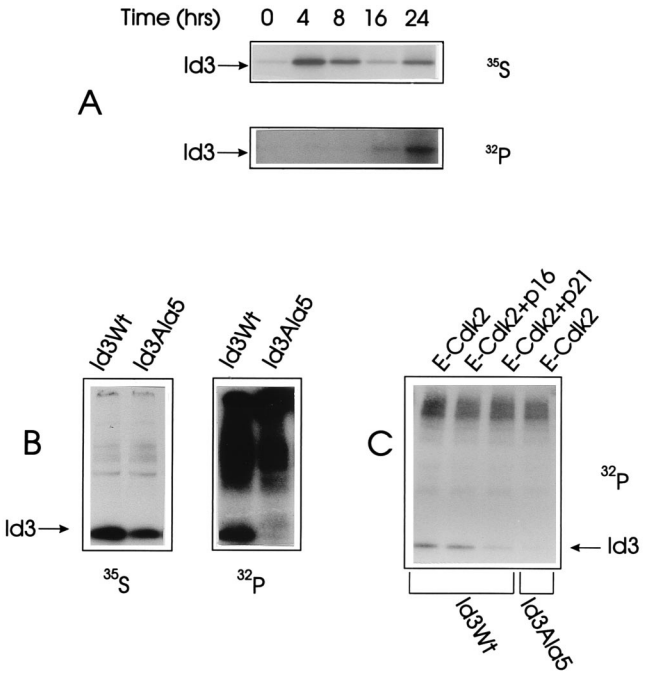

FIG. 2. In vivo phosphorylation of Id3 by cyclin A- and E-Cdk2 complexes. (A) Primary human B cells were stimulated with PMA for the times indicated and then radiolabelled with either [^{35}S]methionine or [^{32}P]orthophosphate. Id3 protein was immunoprecipitated from cell lysates and analyzed by SDS-PAGE. (B) COS cells were transiently transfected with expression vectors encoding either wild-type (Wt) or mutant Id3 protein (Ser5-Ala5) and then radiolabelled with either [^{35}S]methionine or [^{32}P]orthophosphate prior to analysis by immunoprecipitation as described above. (C) COS cells were transfected with either wild-type or Ala5 mutant Id3 expression constructs in combination with expression vectors encoding cyclins, Cdk2, and Cdk2 inhibitors, as indicated. Cells were labelled with [^{32}P]orthophosphate and then lysed and analyzed by immunoprecipitation and SDS-PAGE.

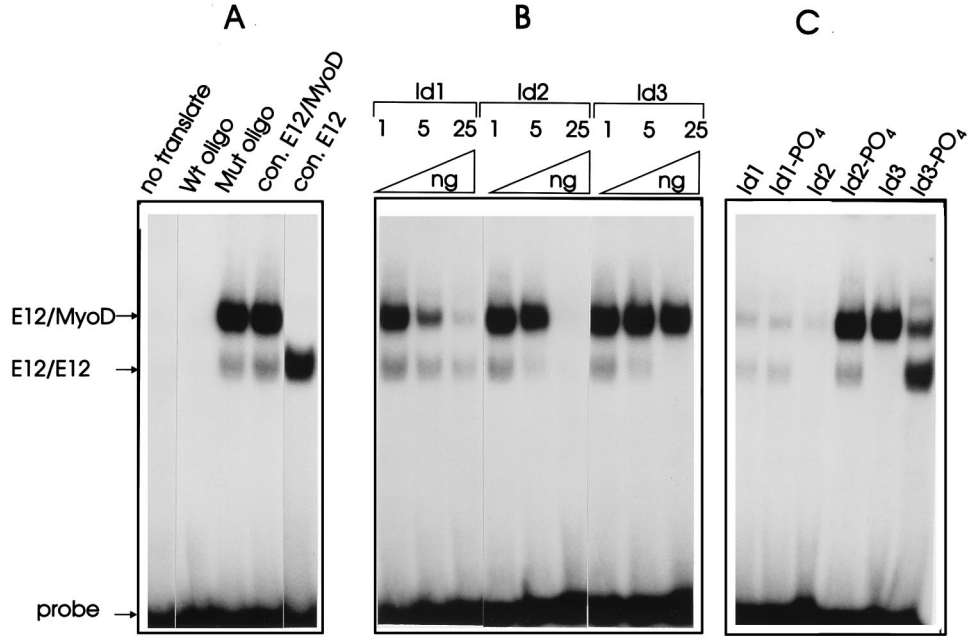

FIG. 3. Attenuation of Id function by phosphorylation at Ser5. Band shift analyses were performed with a ^{32}P-labelled E box oligonucleotide probe together with in vitro-translated E12 and MyoD proteins. The retarded complexes corresponding to the E12 homodimer (con. E12) and to the E12-MyoD heterodimer (con. E12/MyoD) are indicated by arrows; the specificity of interaction was confirmed by competition with a 50-fold excess of unlabelled wild-type (Wt) or mutant (Mut) oligonucleotide probe (A). Complexes were challenged with increasing inputs of each histidine-tagged Id protein (B) or with 25 ng of each Id protein either unphosphorylated or in vitro-phosphorylated with a cell extract from Sf9 cells expressing baculovirus-encoded cyclin A-Cdk2 (C).

Id3 protein. A control Id3 mutant which incorporates a helix-breaking proline residue (Pro49) in the H1 domain (8) was almost totally ineffectual in inhibiting either homodimer or heterodimer bHLH binding (Fig. 4). These results were confirmed in band shift assays evaluating E12 homodimer binding in isolation (Fig. 5).

Effects of phosphorylation on in vivo gene expression and biological properties of Id3. To evaluate the in vivo consequences of Id3 phosphorylation at serine 5, we used constructs expressing wild-type Id3 or the phosphorylation-mimicking mutant (Asp5) to challenge E12-MyoD transactivation of an E-box-dependent reporter gene. As shown in Fig. 6, increasing inputs of wild-type Id3 significantly inhibited transactivation of the E-box-dependent reporter. This contrasts with the lack of inhibition of heterodimer complex seen in band shift assays (Fig. 3 and 4) and can be explained by the exogenous Id3 protein becoming partially phosphorylated in transfected cells (Fig. 2B). However, in agreement with the in vitro band shift data above, the Id3 Asp mutant was much more potent than wild-type Id3, whereas the Pro49 (helix-breaking) mutant had only a marginal effect on reporter gene function (Fig. 6).

To further establish that this enhanced activity of the Id3 Asp mutant in inhibiting E-box-dependent reporter function is due to increased potency in inhibition of E12-MyoD interaction in vivo, we performed a competitive Gal4-Vp16 two-hybrid assay, as shown in Fig. 7. In response to increasing inputs of the wild-type Id3 expression vector, the magnitude of Gal4E12-Vp16MyoD interaction was significantly inhibited, consistent with the ability of the wild-type protein to inhibit E-box-dependent reporter gene activation (Fig. 6). The Pro49 helix-breaking Id3 mutant displayed no significant inhibitory activity. However, the Id3 Asp5 mutant was again more potent than wild-type Id3 at disrupting E12-MyoD heterodimer formation in vivo.

Enforced ectopic expression of Id3 under appropriate conditions promotes cell growth and allows cells to continue cycling under conditions of low serum (1). We therefore used this as an end point to evaluate the possible biological significance of phosphorylation of Id3 at serine 5. Expression constructs encoding wild-type Id3 and phosphorylation-mimicking and phosphorylation-ablating mutants of Id3 were transfected into rat embryo fibroblasts along with a LacZ marker. The cells were then placed in low serum and subsequently pulsed with

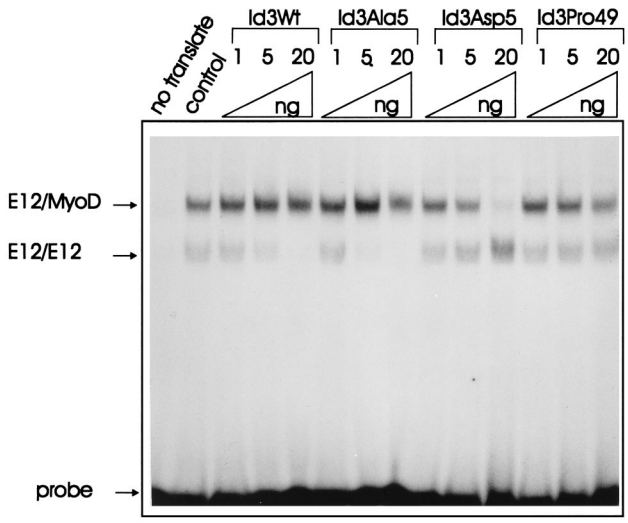

FIG. 4. Effects of phosphorylation-mimicking and phosphorylation-ablating Id3 mutants on E12-MyoD heterodimer bHLH-E box binding. Band shift analysis was performed as described in the legend to Fig. 3, and complexes were challenged with increasing inputs of bacterially synthesized wild-type (Wt) Id3 or the Ala5, Asp5, or Pro49 mutant.

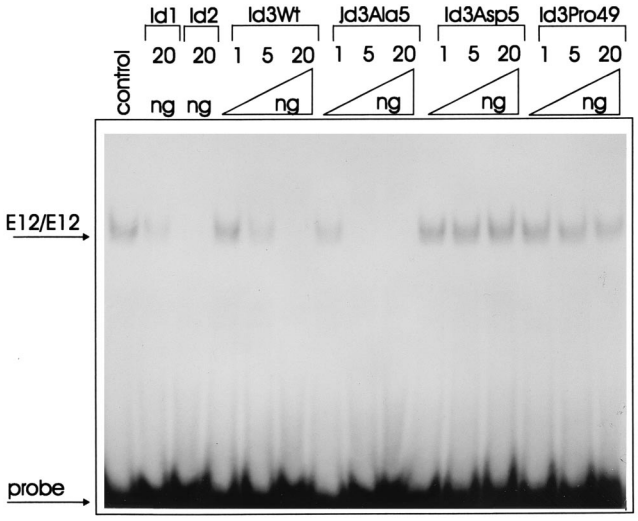

FIG. 5. Effects of phosphorylation-mimicking and phosphorylation-ablating Id3 mutants on E12 homodimeric bHLH-E box binding. Band shift analysis was performed as described in the legend to Fig. 3, and complexes were challenged with increasing inputs of bacterially synthesized wild-type (Wt) Id3 or the Ala5, Asp5, or Pro49 mutant.

BrdU to determine the proportion of cells that remained in cycle. As shown in Fig. 8, approximately 15% of the Id3-transfected cells incorporated BrdU, compared to only 3% of the vector-only transfected controls. The proportion of cycling cells was even higher with the nonphosphorylatable Ala5 mutant (23%), although this effect was accompanied by a degree of cytotoxicity (data not shown). In contrast, the Asp5 and Pro49 mutants of Id3 had only a marginal effect on the proportion of cycling cells relative to the control.

DISCUSSION

Previous studies have shown that during the transition from G_0 to S phase of the cell cycle, the levels of bHLH proteins, exemplified by E2A-related E proteins, remain essentially constant (12, 25). However, during early G_1 the activities of bHLH proteins, as assessed by the binding of cellular protein extract to E box oligonucleotides in vitro, are transiently depressed at a time coincident with the peak of induction of Id proteins (12, 25). In exponentially growing cells, most of the cellular E2A-related protein is associated with Id (17), and in most cell types different Id proteins are typically coexpressed (4, 13). Since Id proteins display both qualitative and quantitative differences in target bHLH protein specificity (reference 12 and as reported herein), it is likely that they act cooperatively to transiently abrogate the function of multiple (as yet uncharacterized) bHLH proteins as an essential mechanism of early G_1 cell cycle progression. However, the expression of Id proteins persists into late G_1 and throughout S phase, and indeed a second peak of induction is seen during late G_1-to-S phase (13), suggesting a functional role beyond early G_1. Enigmatically, the cyclin A– and E–Cdk2-dependent phosphorylation of Id2 at late G_1 was previously found to completely inactivate the function of this Id protein in antagonizing homodimer and heterodimer binding to an E box oligonucleotide in vitro (12). While this loss of Id2 activity might well explain the restoration of bHLH E box binding activity seen in cellular extracts at late G_1 in these experiments, it also cast some doubt on the functional relevance of this phosphorylated Id2 protein. Because of the fortuitous differences in bHLH-antagonizing properties between Id2 and the related Id3 protein, we have now been able to show that analogous cyclin A– and E–Cdk2-dependent cell cycle phosphorylation of Id3 does in fact change its target bHLH specificity. Whereas unphosphorylated Id3 abrogates an E12 homodimer complex but not the E12-MyoD heterodimer, phosphorylation at serine 5 or substitution of a highly charged aspartate residue at this position results in a switch to abrogation of the E12-MyoD heterodimer complex. Moreover, the Id3 Asp5 mutant inhibited E12-MyoD transactivation of an E-box-driven reporter and Gal4E12-Vp16MyoD interaction in vivo more efficiently than the wild-type protein, although we were unable to evaluate each bHLH protein partner in isolation since the respective homodimers gave only a minimal transactivation of the reporters in these assays (reference 22 and unpublished observations). These differences could not be explained purely on the basis of expression levels or by differences in Id protein stabilities, which were comparable in control transfection experiments (data not shown). This phosphorylation-dependent modulation of Id protein specificity is somewhat reminiscent of that reported for phosphorylation of E2A and MyoD (26, 38) and serves to emphasize the role of determinants mapping outside of the bHLH domain in determining the dimerization specificity of Id proteins, as previously implied from molecular modelling studies (43). In this regard, it is noteworthy that the N-terminal domains of both the Id2 and Id3 proteins contain a flexible hinge region between amino acids 20 and 35 that would permit juxtaposition of the serine 5

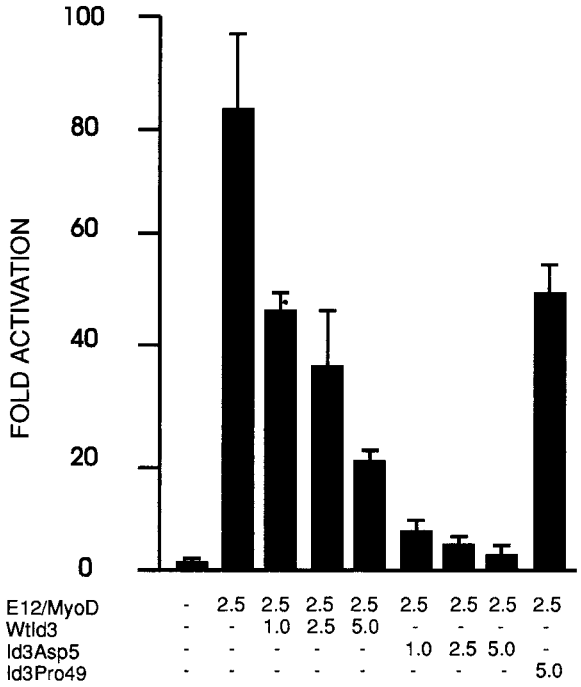

FIG. 6. Enhanced activity of the Id3 phosphorylation mimic on E12–MyoD-dependent gene expression in vivo. Fibroblasts were transfected with 2.5 μg of E-box-driven CAT reporter plasmid in combination with expression vectors encoding E12-MyoD, wild-type (Wt) Id3, Id3 Asp5, or Id3 Pro49, as indicated. Transfected DNA was normalized to a total of 15 μg with pcDNA3 carrier plasmid together with 1 μg of luciferase reporter driven by a heterologous cytomegalovirus promoter as a control for transfection efficiency. Twenty-four hours posttransfection, cells were harvested and relative CAT activities were determined by using [^{14}C]chloramphenicol and thin-layer chromatography with quantitation by phosphoimage analysis. The data shown were determined from two separate experiments.

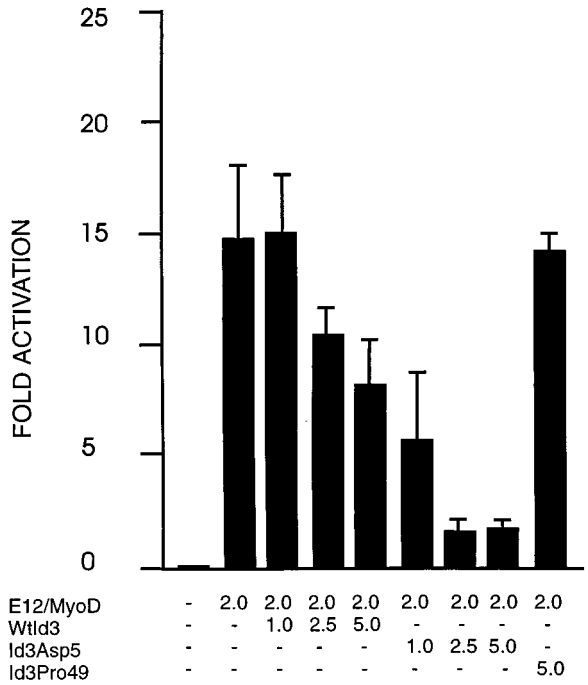

FIG. 7. Enhanced inhibition of Gal4E12-Vp16MyoD interaction in vivo by the Id3 phosphorylation-mimicking Asp5 mutant. Cells were transfected with 1 μg each of the Gal4 luciferase reporter pG5E1bLuc, the cytomegalovirus (CMV)-driven Renilla luciferase expression vector pRLCMV, and the pGal4E12 and pVp16MyoD fusion vectors, together with each of the CMV-driven expression constructs indicated plus pcDNA3 empty vector to normalize for DNA input. At 24 h posttransfection, luciferase activities were measured. The results shown are from two independent experiments. Wt, wild type.

phosphorylatable residue with the respective HLH regions of these Id proteins.

Although these studies utilizing model bHLH proteins dramatically illustrate the consequences of cyclin A– and E–Cdk2-dependent phosphorylation of Id proteins on their functional properties, we have no information on how this relates to their in vivo cellular bHLH target proteins during late G_1 and S phase. This clearly represents an important area for future investigation. Given the rapidly expanding number of reported bHLH transcription factors (24), it is highly probable that phosphorylation of Id2, as with Id3, results in acquisition of altered target bHLH specificity rather than complete loss of bHLH functional antagonism, as previously observed in in vitro bandshift assays (12).

Since substitution of a phosphorylation-mimicking Asp residue at Ser5 in Id3 faithfully reproduced the properties of the phosphorylated Id3 protein seen in in vitro band shift assays and imparted a similar shift towards functional antagonism of bHLH heterodimer-dependent gene expression in vivo, we were able to use this mutant to address the functional significance of Cdk2-dependent phosphorylation at a biological level. In several reported studies, enforced ectopic expression of Id genes has been shown to promote cell growth and to delay exit from the cell cycle on removal of mitogens; this represents an important mechanism through which Id proteins arrest cell differentiation (1, 10, 15). The Asp5 Id3 mutant was completely devoid of any activity in promoting S phase, implying that Cdk-dependent phosphorylation inactivates the G_1-to-S cell cycle regulatory function of this Id protein. Interestingly, the Ala5 Id3 variant imparted a "gain-of-function" phenotype associated with extensive cell death (a property which also precluded analysis of reporter gene function with this mutant). In further experiments, we have observed a similar though less dramatic effect with an Id2 Ala5 mutant (unpublished observations). Previously, the Id2 Ala5 mutant was found to display cell toxicity, since it significantly reduced colony-forming efficiency in stable transfectants (12). The exact nature of the acute cell death induced by Id3 (and Id2) Ala5 mutants revealed in the present study is intriguing, although it does not appear to involve induction of a classical apoptotic phenotype and more likely is the result of an alternative "cell suicide" mechanism (11). It should be noted that most of the Id3 Ala5-transfected cells displaying features of cell death were also in S phase (data not shown). The increased percentage of transfected cells in S phase seen with this mutant (Fig. 8) is therefore not simply attributable to selective killing of cells that are not entering S phase. Indeed, the death of Id3 Ala5-transfected cells may have led to an underestimation of the ability of this mutant to promote S phase. With the caveat that these transfection experiments generate nonphysiological levels of Id proteins, we suggest that Cdk2-dependent phosphorylation of Id2 and Id3 proteins during late G_1 is essential for progression through, or exit from, S phase of the cell cycle. While the phosphorylated Id proteins are nonfunctional in promoting cell growth, they nonetheless antagonize E-box-dependent gene expression, albeit with altered specificity. Thus, while unphosphorylated Id protein may target a set of bHLH proteins (and possibly other proteins) important for regulating G_1 progression, their phosphorylated counterparts may antagonize a distinct set of bHLH transcription factors that are perhaps more important in regulating differentiation-linked gene expression. In addition, these data illustrate that the cell cycle regulatory functions of Id proteins in promoting S phase entry (at least as illustrated for Id3) may be dissociated from the ability to modulate bHLH heterodimer-dependent gene expression. This further underscores the analogy between Ids and their opposing bHLH partners, in which these functional

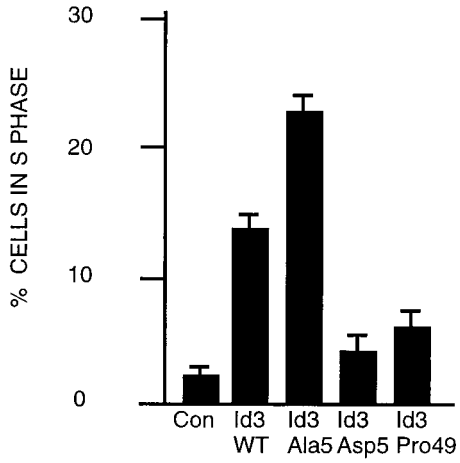

FIG. 8. Id3 mutants selectively promote cell cycle S phase in serum-starved fibroblasts. Rat embryo fibroblasts were transiently cotransfected with the indicated Id3 expression constructs (wild type [WT] or mutant) in combination with an expression construct encoding a *lacZ* marker gene. Control cells (Con) were transfected with the *lacZ* marker gene alone. Twenty-four hours posttransfection, cells were serum starved (0.1% FCS) and, after a further 24 h, were pulse labelled with BrdU. Cells were fixed, and *lacZ*-positive cells were scored by immunofluorescence in combination with detection of BrdU by use of a commercial immunofluorescence detection kit (Boehringer). Results are expressed as percentages of *lacZ*-positive cells appearing in S phase and are taken from two experiments in which a minimum of 200 *lacZ*-positive cells were evaluated for each transfection.

attributes have also been shown to be dissociable (33). Mutants of bHLH proteins that are attenuated in the capacity to activate differentiation-linked gene expression are still functional in promoting cell cycle arrest (7, 40).

The G_0-to-S phase transition in mammalian cells is regulated through the sequential and concerted actions of Cdk's and their regulatory partners, which culminate in phosphorylation of pRB and related pocket proteins, thereby alleviating inhibition of E2F and DP family transcription factors, whose activities are required for further progression through the cell cycle (5, 16, 19, 21, 27). However, a number of other signalling molecules, typically encoded by early response genes such as the Id proteins, are also required for G_0-to-S phase cell cycle progression (28). Understanding how the functions of these seemingly disparate signalling molecules are integrated into the Cdk-coupled Rb-E2F mitogenic cascade represents a pressing challenge. The present study establishes at least one functionally important link between cyclin-dependent kinases and Id-bHLH regulation of cell cycle progression, which also impinges on mechanisms underlying the control of differentiation in the determination of cell fate.

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