Cdk2-dependent phosphorylation of Id2 modulates activity of E2A-related transcription factors

Eiji Hara, Marcia Hall and Gordon Peters¹

Imperial Cancer Research Fund Laboratories, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

¹Corresponding author

The helix–loop–helix (HLH) protein Id2 is thought to affect the balance between cell growth and differentiation by negatively regulating the function of basic-helix–loop–helix (bHLH) transcription factors. Id2 acts by forming heterodimers that are unable to bind to specific (E-box) DNA sequences. Here we show that this activity can be overcome by phosphorylation of a serine residue within a consensus target site for cyclin-dependent kinases (Cdks). *In vitro*, Id2 can be phosphorylated by either cyclin E-Cdk2 or cyclin A-Cdk2 but not by cyclin D-dependent kinases. Analogous phosphorylation occurs in serum-stimulated human diploid fibroblasts at a time in late G₁ consistent with the appearance of active cyclin E-Cdk2. The phosphorylation of Id2 in these cells correlates with the restoration of a distinct E-box-dependent DNA-binding complex, suggesting that the levels of this complex are modulated by both the abundance and phosphorylation status of Id2. These data provide a link between cyclin-dependent kinases and bHLH transcription factors that may be critical for the regulation of cell proliferation and differentiation.

Keywords: cell cycle/cyclin-dependent kinases/E-box/helix–loop–helix proteins/Id proteins

Introduction

The basic-helix–loop–helix (bHLH) proteins are a family of eukaryotic transcription factors that have been shown to play a key role in the differentiation of a number of cell lineages, including muscle cells, B- and T-lymphocytes, pancreatic β cells, osteoblasts and neurons (reviewed in Jan and Jan, 1993; Weintraub, 1993; Olson and Klein, 1994). These proteins generally contain a helix–loop–helix (HLH) dimerization domain, consisting of highly conserved amphipathic helices separated by a loop of variable length and sequence, and an adjacent DNA-binding domain which is rich in basic amino acids (Murre *et al.*, 1989a,b; Davis *et al.*, 1990; Ellenberger *et al.*, 1994; Ma *et al.*, 1994). There are two main categories of bHLH proteins. The so-called A class are ubiquitously expressed proteins such as those encoded by differentially spliced transcripts from the E2A (E12, E47 and E2-5/ITF1 proteins), E2-2/ITF2 and HEB/HTF4 genes (Murre *et al.*, 1989b; Henthorn *et al.*, 1990; Zhang *et al.*, 1991; Hu *et al.*, 1992; Skerjanc *et al.*, 1996) whereas class B comprises tissue-specific bHLH proteins such as the MyoD

(Olson, 1990; Weintraub *et al.*, 1991), NeuroD (Lee *et al.*, 1995; Naya *et al.*, 1995), MASH (Johnson *et al.*, 1990), HAND (Srivastava *et al.*, 1995) and TAL (Begley *et al.*, 1989; Mellentin *et al.*, 1989; Chen *et al.*, 1990) families. Dimerization is essential for DNA binding and transcriptional activity *in vivo* (Lassar *et al.*, 1991) and in general, tissue-specific bHLH proteins form heterodimers with the ubiquitously expressed bHLH proteins, although the latter can also operate as homodimers (Shen and Kadesch, 1995). The basic region of each protein is required for binding to DNA, commonly to a region that includes a specific sequence motif known as the E-box (CANNTG) (Ephrussi *et al.*, 1985; Kiledjian *et al.*, 1988; Lassar *et al.*, 1989) or the related N-box (CACNAG) (Klamt *et al.*, 1989; Tietze *et al.*, 1992).

One additional category of HLH protein has been identified, typified by the four known members of the Id family in mammalian cells, Id1–Id4 (Benezra *et al.*, 1990; Christy *et al.*, 1991; Sun *et al.*, 1991; Biggs *et al.*, 1992; Ellmeier *et al.*, 1992; Deed *et al.*, 1993; Hara *et al.*, 1994; Riechmann *et al.*, 1994; Pagliuca *et al.*, 1995). Analogous proteins have been identified in *Xenopus laevis* (Wilson and Mohun, 1995; Zhang *et al.*, 1995) and *Drosophila melanogaster* (Ellis *et al.*, 1990; Garrell and Modolell, 1990). These resemble bHLH proteins in that they have highly homologous HLH domains but they lack the basic, DNA-binding region. Thus, Id proteins act as negative regulators of the bHLH transcription factors by forming heterodimers that are unable to bind DNA (Benezra *et al.*, 1990; Christy *et al.*, 1991; Sun *et al.*, 1991).

In addition to this ability to inhibit DNA binding, the name Id is often taken to reflect the potential role of these proteins in inhibiting differentiation. For example, the expression of Id genes is down-regulated upon differentiation in many cell types (Benezra *et al.*, 1990; Hara *et al.*, 1991; Sun *et al.*, 1991; Kreider *et al.*, 1992; Le Jossic *et al.*, 1994; Einarson and Chao, 1995). Conversely, ectopic expression of Id1 inhibits B-cell development (Sun, 1994) and the differentiation of muscle (Jen *et al.*, 1992), myeloid (Kreider *et al.*, 1992), erythroid (Shoji *et al.*, 1994; Lister *et al.*, 1995) and mammary epithelial cells (Desprez *et al.*, 1995). Several lines of evidence also suggest that Id proteins play a role in the G₀ to S phase transition of the cell cycle. Thus, the stimulation of quiescent fibroblasts with serum or growth factors induces the transcription of Id1, Id2 and Id3 (Benezra *et al.*, 1990; Christy *et al.*, 1991; Deed *et al.*, 1993; Barone *et al.*, 1994; Hara *et al.*, 1994); inhibiting Id protein synthesis by antisense oligonucleotides prevents the re-entry of arrested cells into the cell cycle (Barone *et al.*, 1994; Hara *et al.*, 1994); and Id expression declines as senescent fibroblasts lose their proliferative activity (Hara *et al.*, 1994).

In mammalian cells, the G₀ to S phase transition is orchestrated by a number of cyclin-dependent kinases

(Cdks), including Cdk4 and Cdk6, acting in conjunction with their regulatory partners, cyclins D1, D2 and D3, and Cdk2 in conjunction with cyclins E and A (reviewed in Peters, 1994; Sherr, 1994; Weinberg, 1995). The current view is that the cyclin D-dependent kinases are essential for passage through the restriction point in late G₁, at which cells become committed to proceed with division irrespective of mitogenic stimuli, whereas cyclin E-Cdk2 activity is required for the initiation of S phase (Hatakeyama *et al.*, 1994; Ohtsubo *et al.*, 1995; Resnitzky and Reed, 1995). There is therefore a great deal of interest in identifying specific substrates for these Cdk complexes. To date, the only known targets for the G₁-specific Cdks are the retinoblastoma gene product (pRb) and its relatives, p107 and p130 (Dowdy *et al.*, 1993; Ewen *et al.*, 1993; Matsushime *et al.*, 1994; Meyerson and Harlow, 1994; Beijersbergen *et al.*, 1995), which serve as negative regulators of heterodimeric transcription factor complexes containing members of the E2F and DP families (Lam and La Thangue, 1994; Weinberg, 1995). Phosphorylation by Cdks appears to be critical for modulating the interactions between these proteins.

Here, we establish a potential link between Cdk activity and bHLH transcription factor complexes by demonstrating that Id2 is a substrate for cyclin E-Cdk2 and cyclin A-Cdk2. Phosphorylation of a specific serine residue within a consensus Cdk target sequence prevents Id2 from interfering with the formation of E-box DNA-binding complexes *in vitro*. Moreover, in serum-stimulated human diploid fibroblasts, the phosphorylation of Id2 occurs at a time in late G₁ that correlates with activation of cyclin E-Cdk2 and with the formation of a specific DNA-binding complex that would otherwise be prevented by the excess of Id2. The data suggest that cell cycle-dependent modulation of bHLH activity may be achieved by regulating the abundance and phosphorylation status of Id proteins

Results

E-box DNA-binding complexes in serum-stimulated human fibroblasts

The complementary effects of E2A and Id proteins on cell proliferation prompted us to ask whether the DNA-binding complexes formed on a typical E-box (CANNTG) varied during cell cycle progression. Human diploid fibroblasts (TIG-3 cells) were rendered quiescent in medium containing 0.2% serum for 4 days and subsequently induced to re-enter the cell cycle by addition of 20% serum. Nuclear extracts were prepared at various times after addition of serum and used in electrophoretic mobility shift assays (EMSA) with an oligonucleotide probe from the muscle creatine kinase (MCK) enhancer. This oligonucleotide has been used extensively to characterize bHLH protein complexes in a variety of cell types (Johnson *et al.*, 1992; Kreider *et al.*, 1992; Einarson and Chao, 1995). A high molecular weight protein-DNA complex was observed whose abundance changed significantly during the progression from G₀ to S phase (Figure 1A). Relatively low levels of this complex were formed with extracts from quiescent cells (lane 1) but it became barely detectable after 4 h of serum stimulation (lane 2). However, the complex was re-established by 12 h and reached a maximum between 16 and 24 h after serum

Fig. 1. Cell cycle-dependent expression of an E-box complex sensitive to Id2. Quiescent TIG-3 cells were stimulated by the addition of serum and nuclear extracts or total cell lysates were prepared at the indicated times after serum addition. **(A)** The nuclear extracts were used in EMSA assays using a 26 bp oligonucleotide E-box probe from the muscle creatine kinase (MCK) promoter. The specific high molecular weight complex (arrowed) was competed by excess oligonucleotide (lane 9) but not by mutant oligonucleotide (lane 8). In lanes 10 and 11, 20 ng of bacterially expressed Id1 or Id2 were added to the reactions. **(B)** Samples (50 μ l) of total cell lysate were fractionated by SDS-PAGE in a 12% acrylamide gel, transferred to nitrocellulose, and immunoblotted with antisera against human E2A, Id2 or cyclin A as indicated on the right. The immune complexes were detected by ECL.

addition (Figure 1A). The specificity of the complex was confirmed by its sensitivity to competition by an excess of unlabelled oligonucleotide but not by an equivalent oligonucleotide containing a mutation in the E-box (Figure 1A, lanes 8 and 9). Moreover, the formation of this protein-DNA complex was inhibited by the addition of

Fig. 2. Amino acid sequence alignment of the four human Id proteins. The predicted amino acid sequences of Id1, Id2, Id3 and Id4 are shown in single letter code and regions of similarity are boxed (designated Boxes 1–4). The highly conserved HLH region (Box 2) is depicted by the striped lines. The bold lines in Box 1 identify consensus sites for phosphorylation by Cdk2 that are present in Id2, Id3 and Id4, but not in Id1. The sequences in this figure are based on the data in Biggs *et al.* (1992), Deed *et al.* (1993), Hara *et al.* (1994) and Pagliuca *et al.* (1995).

20 ng of human Id2 protein, expressed as a histidine-tagged fusion protein in bacteria, although a similar quantity of human Id1 protein had no effect (Figure 1A, lanes 10 and 11).

The most obvious explanation for these results would be that the proteins that participate in the complex are expressed at variable levels during the G₀ to S phase transition. However, the prototypic E-box-binding proteins encoded by the E2A gene did not show equivalent cell cycle fluctuations as judged by immunoblotting of cell lysates with an E2A-specific antiserum (Figure 1B). In contrast, it has been previously established that Id gene expression is dramatically increased following serum stimulation of quiescent cells, with two distinct peaks in early G₁ and at the G₁–S transition (Barone *et al.*, 1994; Hara *et al.*, 1994). The biphasic induction of Id2 in serum-stimulated TIG-3 cells is illustrated in Figure 1B. Thus, Id2 protein levels were extremely low in quiescent cells, reached a maximum at about 2–4 h after serum addition and declined again by 8–12 h. This pattern of Id2 expression in early G₁ is the inverse of that of the E-box-binding complex in Figure 1A, suggesting that the level of the complex might be dictated by the expression of Id2. However, the second peak of Id2 expression, which roughly parallels the induction of cyclin A in late G₁ and S phase (Figure 1B), coincides with maximum levels of the E-box complex.

Phosphorylation of Id2 in vitro by cyclin E–Cdk2 and cyclin A–Cdk2

To try to reconcile these observations, we considered whether some of the relevant proteins might be subject to cell cycle-dependent phosphorylation, by analogy with components of the pRb/E2F system of transcriptional regulators. It has been previously noted that some Id proteins contain consensus phosphorylation sites for a variety of kinases, including the cyclin-dependent kinases (Nagata *et al.*, 1995). Figure 2 shows an amino acid sequence alignment for the four Id genes identified in

human cells. In all Id family members characterized thus far in different species, the HLH region is highly conserved (Box 2 in Figure 2). However, the sequences outside the HLH motif are distinct except for three regions that show some degree of homology, designated boxes 1, 3 and 4. In particular, box 1 contains a sequence, SPVR, that conforms to the consensus for phosphorylation by cyclin-dependent kinases. This motif is conserved in Id2, Id3 and Id4 but is not present in the Id1 protein, identifying a potential difference between Id1 and other family members.

To determine whether the Id proteins are indeed targets for phosphorylation by Cdks, human Id1 and Id2 were expressed in bacteria as fusion proteins with glutathione S-transferase (GST) and used as substrates in an *in vitro* kinase assay with different combinations of cyclins and Cdks. The active enzymes were generated by co-expressing the cyclins and Cdks in Sf9 insect cells using baculovirus vectors; previous studies (Kato *et al.*, 1993; Meyerson and Harlow, 1994; Parry *et al.*, 1995) have shown that the cyclin–Cdk complexes generated in this system are capable of phosphorylating relevant substrates, such as pRb, as GST-linked fusion proteins. Several combinations were compared that were representative of the cyclin D-dependent kinases and of Cdk2 complexed to either cyclin E or cyclin A. GST–Id1, which lacks a consensus Cdk target, was not phosphorylated by any of the cyclin–Cdk complexes tested (Figure 3A). In contrast, GST–Id2 protein was clearly phosphorylated by Cdk2 in association with either cyclins E or A (Figure 3B, lanes 7 and 8) but not by the cyclin D-dependent kinases Cdk4 and Cdk6 (Figure 3A, lanes 1–6). The activity of each kinase complex was confirmed by the phosphorylation of GST–Rb, which is a substrate for all of the cyclin–Cdk complexes tested and contains multiple Cdk consensus sites (Figure 3C and additional data not shown). The clear distinction between Id2 which has a single consensus Cdk site and Id1 which does not, suggested that the phosphorylation of Id2 was a specific and potentially relevant event. Moreover, the phosphorylation of both GST–Rb and GST–Id2 by cyclin E–Cdk2 was abolished by addition of the Cdk inhibitor p21^{CDKN1} (Figure 3C), suggesting that it was attributable to a Cdk rather than to non-specific kinase activity in the insect cell lysates.

Effects of phosphorylation on Id2 activity in vitro

To determine whether the phosphorylation of Id2 by Cdk2 alters its activity, an electrophoretic mobility shift assay (EMSA) was established using the MCK E-box probe and components expressed by coupled transcription and translation of plasmid DNAs. The sizes and yields of the various products were verified by SDS–PAGE (not shown). A partial fragment of the E12 protein, ΔE12 (Staudinger *et al.*, 1993), which includes the bHLH region (residues 508–654) formed a complex on the ³²P-labelled DNA probe consistent with the formation of a ΔE12 homodimer (Figure 4A, lane 2). A much weaker band shift was detected using MyoD alone (Figure 4A, lane 3) consistent with its reduced ability to form homodimers as opposed to heterodimers with other HLH proteins. A 1:1 mixture of ΔE12 and MyoD proteins produced an additional DNA-binding complex, presumably a heterodimer, that migrated at an intermediate position between the ΔE12–ΔE12 and

Fig. 3. Phosphorylation of GST-Id2 *in vitro* by cyclin-Cdk2 complexes. Sf9 insect cells, either uninfected or co-infected with baculovirus vectors encoding a cyclin (D1, D2, D3, E or A) and a kinase (Cdk2, Cdk4 and Cdk6) were used to assemble the indicated cyclin-Cdk complexes. The relevant cell lysates were then incubated with GST-Id1 (A) or GST-Id2 (B) in the presence of [γ - 32 P]ATP. Labelled proteins were then analysed by SDS-PAGE in a 12% acrylamide gel and visualized by autoradiography. (C) Cyclin E-Cdk2 complexes were used to phosphorylate GST-Rb, GST-Id1 or GST-Id2 in the presence or absence of the Cdk-inhibitor p21^{CDKN1} expressed as a histidine-tagged fusion protein in bacteria.

MyoD-MyoD homodimer complexes (Figure 4A, lane 4). As controls for the specificity of these interactions, an excess of unlabelled oligonucleotide completely abolished the formation of complexes on the labelled probe whereas an oligonucleotide containing a mutated E-box sequence did not (Figure 4A, lanes 5 and 6). Moreover, addition of a polyclonal antiserum against MyoD decreased the mobility of the MyoD-MyoD complexes (Figure 4A, lanes 7 and 8) as well as affecting the mobility of the presumed Δ E12-MyoD heterodimer (Figure 4A, lanes 9 and 10). A higher concentration of antibody (2 μ l) was required to shift the heterodimer whereas the Δ E12- Δ E12 homodimer complex was unaffected by the MyoD antiserum.

Having validated the mobility shift assay, we next asked whether the addition of Id proteins could interfere with the formation of complexes containing Δ E12- Δ E12 homodimers or Δ E12-MyoD heterodimers. Approximately 0.5–

1 ng of recombinant Id1 protein was enough to inhibit the formation of a Δ E12-MyoD-DNA complex (Figure 4B, lanes 3 and 4) whereas 5- to 10-fold more Id2 protein (~5 ng) was required to obtain the same effect on the heterodimer (Figure 4B, lane 11). In contrast, the addition of between 10 and 20 ng of Id2 protein was able to inhibit the formation of the homodimer Δ E12- Δ E12-DNA complex (Figure 4B, lanes 12 and 13) whereas disruption of the homodimer complex was not observed with Id1 protein (lane 7). These results indicate a hitherto unrecognized difference in the specificity of Id1 and Id2 for binding to bHLH proteins.

Similar competition assays were then performed using preparations of Id1 and Id2 that had been pretreated with Sf9 cell lysates containing active cyclin A-Cdk2 and unlabelled ATP (Figure 4C, lanes 6 and 10) or incubated with uninfected Sf9 cell lysates (Figure 4C, lanes 3–5 and 7–9). Increasing amounts of the Id1 and Id2 proteins that had been treated with uninfected Sf9 cell lysates inhibited the formation of Δ E12-MyoD-DNA complexes as before (lanes 3–5 and 7–9) whereas addition of 10 ng of an unrelated histidine-tagged protein, in this case p16^{CDKN2a}, had no effect (lane 2). Predictably, incubation of Id1 with cyclin A-Cdk2 made no discernible difference to its activity in this assay, since Id1 lacks the relevant Cdk target site (Figure 4C, lane 6). However, incubation of Id2 with cyclin A-Cdk2 completely abolished its ability to disrupt the Δ E12- Δ E12 and Δ E12-MyoD DNA-binding complexes, even with 10 ng of protein (Figure 4C, lane 10). These data imply that Id2 is inactivated by phosphorylation by cyclin A-Cdk2.

Identification of the Cdk2 phosphorylation site in Id2

As shown in Figure 2, there is a potential phosphorylation site for Cdk-type kinases close to the amino-terminus of the human Id2 protein. This site conforms to the well-established consensus sequence for Cdk substrates (S/T-P-X-K/R) and is conserved in the sequences of Id3, Id4 and *Xenopus* Id-proteins (Wilson and Mohun, 1995; Zhang *et al.*, 1995). To determine whether this site is indeed the Cdk2 target, a mutant form of Id2 was generated (designated Id2-S5A) in which the relevant serine was changed to alanine. The Id2-S5A mutant was not detectably phosphorylated by cyclin A-Cdk2 *in vitro* (Figure 5A). In addition, bacterially produced Id2-S5A protein remained capable of inhibiting the formation of a Δ E12-MyoD-DNA complex both before (Figure 5B, lane 2) and after treatment with cyclin A-Cdk2 (Figure 5B, lane 3). This contrasts with the behaviour of the wild-type Id2 protein in the same assay (Figure 5B, lanes 4 and 5). These data suggest that phosphorylation of Ser5 is important for the regulation of Id2 activity.

Phosphorylation of Id2 during G₀ to S phase progression

To determine whether an analogous phosphorylation of Id2 occurs *in vivo*, quiescent TIG-3 human diploid fibroblasts were induced to re-enter the cell cycle by addition of serum and at various times thereafter the cells were incubated with 32 P-labelled orthophosphate for 2 h and immunoprecipitated with a polyclonal antiserum against Id2. As shown in Figure 6A, the Id2 protein

became ^{32}P -labelled at ~12 h following serum addition and continued to be phosphorylated at subsequent time points up to 24 h. Flow cytometric analysis of parallel cell cultures indicated that the cells began to enter S phase after ~16 h.

The timing of this phosphorylation would be more consistent with the action of cyclin E-Cdk2 than cyclin A-Cdk2 (Dulic *et al.*, 1992; Koff *et al.*, 1992). To address this issue, equivalent cultures of unlabelled TIG-3 cells were precipitated with antisera against either cyclin E or cyclin A and the associated kinase activities assayed on histone H1 as substrate (Figure 6B). The cyclin E-dependent H1-kinase activity was first detectable at ~12 h after serum addition, correlating well with the first appearance of phosphate label in Id2. In contrast, the most significant increase in cyclin A-dependent H1-kinase activity did not begin until between 18 and 24 h after serum stimulation. However, these data do not exclude a contribution from both complexes.

It was also important to determine whether this *in vivo* phosphorylation of Id2 protein occurred on the same site or sites as the *in vitro* phosphorylation by cyclin-Cdk complexes. The ^{32}P -labelled Id2 bands from metabolically labelled cells were therefore fractionated by SDS-PAGE, recovered from the gels and subjected to two-dimensional tryptic peptide mapping (Boyle *et al.*, 1991). Similar maps were prepared using histidine-tagged Id2 protein that had been phosphorylated in Sf9 cell lysates by cyclin A-Cdk2. A single tryptic phosphopeptide was observed in Id2 that had been phosphorylated *in vitro*, whereas two phosphopeptides were observed in the Id2 recovered from labelled cells (Figure 7A and B). In a mixing experiment, it was confirmed that the tryptic peptide labelled *in vitro* by cyclin A-Cdk2 co-migrated with one of the peptides labelled *in vivo* (indicated by the arrow in Figure 7C). These results indicated that the Id2 protein is phosphorylated on Ser5 *in vivo*. The identity of the other phosphopeptide is presently unclear but it presumably reflects the action of a non-Cdk kinase. For example, there is a report that Id2 can be phosphorylated by PKA or PKC *in vitro*, but that neither phosphorylation affects the activity of Id2 (Nagata *et al.*, 1995). In our experiments, both Id2 peptides became phosphate-labelled by 12 h after serum addition and the ratios of the two spots remained essentially constant at subsequent time points (not shown).

Fig. 4. Effects of phosphorylation on the activity of Id2. (A) The ΔE12 form of E12 and MyoD were synthesized by *in vitro* translation and tested for the ability to bind to the MCK promoter E-box oligonucleotide in an electrophoretic mobility shift assay. The positions of the free ^{32}P -labelled probe and the homo- and heterodimeric complexes are indicated on the right. Lane 1 shows the rabbit reticulocyte lysate control and lanes 2–10 contain the indicated combinations of ΔE12 and MyoD. In lane 5, an excess of the unlabelled oligonucleotide probe and in lane 6, a mutated version of the oligonucleotide were added as a competitor. In lanes 7–10, either 0.2 or 2.0 μl of MyoD antiserum was added to the reaction mixture. (B) A similar assay using ΔE12 and MyoD together with 0, 0.1, 0.5, 1.0, 5, 10 or 20 ng of bacterially expressed, histidine-tagged Id1 or Id2. (C) An analogous assay using preparations of histidine-tagged Id1 or Id2 that had been preincubated with uninfected Sf9 cell lysate (lanes 3–5 and 7–9) or with lysate containing active cyclin A-Cdk2 complex (lanes 6 and 10). The different amounts of protein added are indicated above each lane. Lane 2 shows a reaction to which 10 ng of histidine-tagged p16^{CDKN2a} had been added as a non-specific control.

Fig. 5. Identification of the Cdk2 phosphorylation site in Id2. (A) Histidine-tagged versions of Id1, Id2 and the Id2-S5A mutant were expressed in bacteria, purified on chelating agarose, and tested as substrates for phosphorylation by Sf9 cell lysates containing cyclin A-Cdk2. (B) The Id2-S5A and wild-type Id2 proteins were compared for their ability to disrupt Δ E12-MyoD-DNA complexes before (lanes 2 and 4) and after phosphorylation by Sf9 cell lysates containing cyclin A-Cdk2 (lanes 3 and 5). The positions of the Δ E12-MyoD heterodimer and Δ E12- Δ E12 homodimer complexes are indicated on the right.

Modulation of Id2 activity by phosphorylation in late G₁

Taken together, the *in vitro* and *in vivo* data would be consistent with a model in which the phosphorylation of Id2 by cyclin E-Cdk2 allows the restoration of the E-box-binding complex observed in TIG-3 cells in late G₁. However, the data do not exclude some fluctuation in the levels of E-box-binding proteins other than E2A and do not address the relative abundance of the bHLH and Id2 proteins. To examine these issues and to obtain further support for the model, mobility shift assays were performed using mixtures of nuclear extracts prepared from TIG-3 cells at either 4 or 16 h after addition of serum. The former should contain predominantly unphosphorylated Id2 while the latter should contain essentially the same amount of Id2 protein (see Figure 1B) that has been phosphorylated on Ser5. As shown previously, the 4 h extract did not support the formation of a DNA-binding complex whereas the 16 h extract did (Figure 8, lanes 1 and 2). Significantly, a 1:1 mixture of the two extracts did not give a detectable complex (lane 3) suggesting that the amount of free unphosphorylated Id2 in the 4 h extract was able to compete with all of the available bHLH proteins in the 16 h extract. Decreasing the proportion of 4 h extract only marginally restored the formation of the E-box complex (lanes 4 and 5). More substantial restoration of the complex was achieved by partially depleting the unphosphorylated Id2 in the 4 h extract by immunoprecipitation (lane 6). As predicted, a similar immunodepletion experiment with antiserum against Id1 had no

Fig. 6. Phosphorylation of Id2 protein *in vivo*. (A) Quiescent TIG-3 cells were stimulated by the addition of serum and pulse-labelled for 2 h with [³²P]orthophosphate at various times after serum addition (as indicated). Cell extracts were immunoprecipitated with a polyclonal antiserum against Id2 and fractionated by SDS-PAGE in a 12% acrylamide gel. The labelled Id2 protein was detected by autoradiography. (B) Parallel cultures of stimulated TIG-3 cells were immunoprecipitated with antiserum against human cyclin E or cyclin A and the level of associated kinase activity was assayed using histone H1 as a substrate. The phosphorylated products were analysed by SDS-PAGE, detected by autoradiography, and quantitated by phosphorimaging.

effect on the potency of the 4 h extract. Since the amount of Id2 expressed in late G₁ is equivalent to that in the 4 h sample, there would clearly be enough to block the formation of E-box complexes unless the vast majority of it was prevented from doing so by phosphorylation. We suggest, therefore, that the fluctuations in the E-box complex observed in serum-stimulated TIG-3 cells reflect both the level and phosphorylation status of Id2 and that the reappearance of this complex in late G₁ coincides with the phosphorylation of Id2 by cyclin E-Cdk2.

Inhibition of cell growth by unphosphorylated Id2

Since it has been previously suggested that elevated expression of Id2 stimulates cell growth (Iavarone *et al.*, 1994; Lasorella *et al.*, 1996), we were interested in determining whether the S5A mutant behaved differently. The wild-type and S5A cDNAs were therefore transferred into the eukaryotic expression vector pcDNA3 (which

Fig. 7. Tryptic peptide mapping of phosphorylated Id2. ³²P-labelled Id2 protein phosphorylated *in vitro* using cyclin A-Cdk2 produced in Sf9 cells (A) or immunoprecipitated from metabolically labelled TIG-3 cells (B), was subjected to two-dimensional tryptic peptide mapping. The first dimension was electrophoresis (from left to right) and the second dimension chromatography (bottom to top), with the origin indicated as O. The labelled phosphopeptides were detected by autoradiography. (C) A mixing experiment in which equal amounts of Id2 labelled *in vitro* and *in vivo* were mixed before tryptic peptide mapping. The arrow indicates the major phosphopeptide common to both preparations.

carries a neomycin-resistance gene) and transfected into either NIH 3T3 cells or the U2OS human osteosarcoma cell line. As illustrated in Table I, transfection of wild-type Id2 had no significant effect, relative to the vector-only control, on the number of G418-resistant colonies observed. In contrast, transfection of the S5A mutant caused a 2-fold decrease in the number of colonies. Consistent results were obtained in several experiments and with both NIH 3T3 and U2OS cells (Table I). Although the interpretation of these data is complicated by the presence of endogenous Id2, they clearly imply that the non-phosphorylatable form of Id2 is growth inhibitory. Thus, phosphorylation of Id2 may be required for cells to progress from G₁ into S.

Discussion

There are a number of reasons to suspect a link between Id function and cell cycle progression. In general terms, ectopic expression of Id favours cell proliferation at

Fig. 8. Modulation of E-box-binding complexes in TIG-3 cells by Id2. Electrophoretic mobility shift assays were performed using nuclear extracts prepared from TIG-3 cells at 4 h or 16 h after serum addition (as in Figure 1A). Each reaction contained a total of 5 µl of nuclear extract. In lanes 3, 4 and 5, the 4 h and 16 h extracts were mixed in 1:1, 1:2 and 1:3 ratios respectively (shown as percentage contributions above each lane). In lanes 6 and 7, the 4 h extract was pre-cleared by immunoprecipitation with an antiserum against either Id2 or Id1, as indicated.

Table I. Effects of Id2 and Id2-S5A on cell growth^a

DNA	No. of colonies		
	Experiment 1	Experiment 2	Experiment 3
Vector	184 ± 12	166 ± 14	1730 ± 10
Id2-WT	190 ± 10	157 ± 8	1800 ± 40
Id2-S5A	101 ± 2	82 ± 8	823 ± 42

^aSubconfluent mouse NIH 3T3 cells or human U2OS cells were transfected with pcDNA3 (vector) or with pcDNA³ containing wild-type Id2 (Id2-WT) or the S5A mutant (Id2-S5A). After 16 days in selective medium, the G418-resistant colonies were stained and counted. The results represent the average (± SD) of duplicate 100-mm dishes in three independent experiments. Experiments 1 and 2 used NIH 3T3 cells and experiment 3 used U2OS cells.

the expense of differentiation whereas overexpression of bHLH proteins is associated with cell cycle arrest (Crescenzi *et al.*, 1990; Sorrentino *et al.*, 1990; Jen *et al.*, 1992; Kreider *et al.*, 1992; Deed *et al.*, 1993; Gu *et al.*, 1993; Iavarone *et al.*, 1994; Peverali *et al.*, 1994; Desprez *et al.*, 1995). More specifically, the expression of Id genes is markedly and rapidly increased when quiescent cells are stimulated with serum growth factors (Benezra *et al.*, 1990; Christy *et al.*, 1991; Barone *et al.*, 1994; Hara *et al.*, 1994) whereas abrogation of Id expression using antisense oligonucleotides prevents G₁ progression (Barone *et al.*,

1994; Hara *et al.*, 1994). Since the Id proteins act by forming inactive heterodimers with bHLH transcription factors, we were interested in determining whether cell cycle variations in Id expression were reflected in the types and levels of DNA-binding complexes formed by the bHLH proteins.

We chose to examine this issue in human diploid fibroblasts, which are known to express both Id1 and Id2 as well as E2A-related products, and which respond predictably in classical serum depletion and addition experiments (Hara *et al.*, 1994). Nuclear extracts from these cells contain proteins that form a distinct DNA-binding complex on a standard E-box oligonucleotide from the MCK promoter (Figure 1A). However, we are not certain of the identity of the bHLH proteins in this complex since it was only partially sensitive to E2A antiserum (not shown). Interestingly, the complex was sensitive to competition by Id2 but not Id1 (Figure 1A), indicating a clear difference in the specificity of the Id family members. A similar difference was apparent in the *in vitro* EMSA assays in that Id2 was much more effective than Id1 at disrupting Δ E12 homodimers (Figure 4A and B), as also noted by Nagata *et al.* (1995). Conversely, Id1 was more effective than Id2 at disrupting the formation of Δ E12-MyoD heterodimers. Analogous results were obtained using the full-length E12 protein although the resultant homodimer appeared to bind DNA less effectively than the Δ E12 homodimer (not shown). A previous report suggested that a domain adjacent to the bHLH region in E12 inhibits the DNA-binding capacity of the E12 homodimer (Sun *et al.*, 1991). However, the Δ E12 protein used in the present study (residues 508–654) and another derivative of E12, encompassing amino acids 490–654, were both found to bind efficiently to DNA in this system despite the presence of the putative inhibitory domain (Figure 4 and additional data not shown).

Irrespective of its composition, a striking feature of the E-box complex observed in Figure 1 was its fluctuation during the G₀ to S phase progression. The level of the complex declined markedly upon serum stimulation, with a nadir at ~4 h, but then recovered at ~8–12 h. This pattern was an almost exact mirror image of the initial peak of Id2 expression, suggesting that the induction of Id2 was preventing the formation of the complex. The mixing experiments described in Figure 8 would be consistent with this interpretation as Id2 appeared to be in considerable excess over the bHLH proteins at the 4 h time point. A stoichiometric excess of Id proteins over the E-proteins has been noted in other cell systems (Jen *et al.*, 1992). However, the data in Figure 1 presented an obvious paradox in that the maximum levels of the E-box complex occurred at 16–24 h, at a time when Id2 expression was also maximal.

The resolution of this paradox rests in the phosphorylation of Id2 that takes place in late G₁ (Figure 6). Tryptic peptide mapping confirmed that at least part of this phosphorylation occurs on a peptide that is a target for Cdk2 *in vitro* (Figure 7) and its timing would be consistent with the activation of cyclin E-Cdk2 (Figure 6 and Dulic *et al.*, 1992; Koff *et al.*, 1992). Curiously, the second tryptic phosphopeptide in metabolically labelled Id2 also showed cell cycle dependence, appearing at roughly the same time as (perhaps marginally before) the peptide

containing the Cdk2 site. The location of this second phosphorylation site and its possible significance are not yet clear. In contrast, we showed that Cdk2 phosphorylation occurs on Ser5 (Figure 5), within an obvious consensus for Cdk phosphorylation, SPVR, that is common to Id2, Id3 and Id4 (see Figure 2). We also demonstrated unequivocally that phosphorylation of Ser5 by Cdk2 negates the ability of Id2 to disrupt Δ E12 homodimer and Δ E12-MyoD heterodimer complexes on DNA (Figure 4) whereas the S5A mutant of Id2 remained effective even after exposure to active cyclin A-Cdk2 (Figure 5). Thus, the high levels of both the DNA-binding complex and Id2 can be readily reconciled if the Id2 protein expressed in late G₁ is quantitatively inactivated by phosphorylation by cyclin E-Cdk2.

If this is indeed the case, then what purpose would be served by the second peak of Id2 expression in late G₁? One possibility is that the phosphorylation of Id2 by cyclin E-Cdk2 kinase does not inactivate Id2 but changes its binding specificity. Thus, while E2A-like bHLH proteins may be the main targets of Id2 in early G₁ phase, the Cdk2-phosphorylated form of Id2 that prevails in late G₁ might bind to some other protein that is important for regulating the G₁ to S transition. Perhaps the most attractive candidate would be pRb as it has been reported that pRb binds to Id2, but not to Id1 or Id3 (Iavarone *et al.*, 1994; Lasorella *et al.*, 1996). However, we have been unable to confirm this interaction by co-precipitation from cell lysates, by binding *in vitro*, or by testing the components in a yeast two-hybrid assay (not shown). Another candidate could be the recently identified MIDA1 protein, which lacks a canonical HLH motif yet was isolated via its interaction with Id (Shoji *et al.*, 1995).

To try to address the biological significance of the phosphorylation, we transfected cells with vectors expressing Id2 and the non-phosphorylatable S5A, in the expectation that Id2 might promote cell growth (Iavarone *et al.*, 1994; Lasorella *et al.*, 1996). In our hands, Id2 alone had no effect on the colony-forming efficiency of either NIH 3T3 or U2OS cells (Table I) and did not significantly change the cell cycle profile of transiently transfected U2OS cells (not shown). In hindsight, this is not particularly surprising given that both the exogenous and endogenous Id2 in these cells will presumably be subject to phosphorylation by Cdk2. However, expression of Id2-S5A caused an ~50% reduction in colony formation, suggesting that the continued presence of non-phosphorylated Id2 is growth inhibitory. The simplest interpretation would be that Id2 has to be phosphorylated in order for cells to progress through the cycle, but this is a complex issue that requires further investigation.

Many other questions remain unresolved. Perhaps the most pressing is the identity of the E-box-binding proteins observed in serum-stimulated fibroblasts and experiments are underway to try to characterize these components. Since the complex is sensitive to Id2 but not to Id1, it is interesting to consider whether fibroblasts contain additional bHLH proteins, perhaps binding to distinct E-box elements, that are regulated by Id1. Because Id1 lacks the Cdk consensus site, it may not be inactivated in late G₁ despite showing the same biphasic pattern of expression as Id2. On the basis of the SPVR site, it seems likely that Id3 and Id4 might be subject to the same regulatory

controls as Id2 and indeed Nagata *et al.* showed that rat Id3 could be phosphorylated *in vitro* by a purified preparation of cdc2 (Nagata *et al.*, 1995). Although all the Id proteins have consensus sites for other types of kinase, phosphorylation by PKA within the HLH region of Id1 and Id2 did not demonstrably affect their properties (Nagata *et al.*, 1995). It is therefore intriguing that phosphorylation close to the amino-terminus of Id2 has such a profound effect on binding via the HLH domain, perhaps indicating that this domain (box 1 in Figure 2) either participates in or contributes to the interactions with E-proteins.

Finally, there are several parallels between phosphorylation of Id2 by cyclin E-Cdk2 shown here and the phosphorylation of pRb and related pocket proteins by cyclin D- and cyclin E-dependent kinases. Phosphorylation of Id2 relieves its inhibitory effects on members of the E2A family of transcription factors whereas phosphorylation of pRb and its relatives relieves the inhibition of the E2F family of transcription factors. However, whereas pRb, p107 and p130 are thought to participate in DNA-binding complexes, the Id proteins act by forming heterodimers that are unable to bind to DNA. Nevertheless, in both cases, the action of cyclin-dependent kinases will directly influence the patterns of gene expression during the cell cycle. The pressing challenge is to identify genes relevant to the G₀ to S phase transition that are either positively or negatively regulated by Id-sensitive transcription factor complexes.

Materials and methods

Cell culture and colony assays

Normal human diploid fibroblasts, TIG-3 (Ohashi *et al.*, 1980; obtained from the Japanese Cancer Research Resources Bank, Tokyo, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The TIG-3 cells used in this study were at relatively early passage, with estimated population doublings (PDs) of ~30–35.

For colony-forming assays, human U2OS osteosarcoma cells and mouse NIH 3T3 cells, grown in DMEM plus 10% FBS, were seeded at 50% confluence in 100 mm dishes. The following day, duplicate plates were transfected with 10 µg of pcDNA3 (Invitrogen), pcDNA3-Id2-WT, or pcDNA3-Id2-S5A plasmid DNAs using the lipofectamine reagent as described by the supplier (Gibco-BRL). After a further 24 h, the cells were split 1:15 in medium containing 350 µg of G418/ml. The medium was changed every 4 days and after 16 days the cells were fixed and stained with Giemsa.

Purification of bacterially expressed Id proteins

The human Id1 and Id2 coding sequences (Hara *et al.*, 1994) were cloned into the pGEX-T vector (Pharmacia) or the pRSET-B vector (Invitrogen) for expression in bacteria. The recombinant proteins were expressed in *Escherichia coli* BL21(DE3)pLysS. GST-Id proteins were purified according to published protocols (Smith and Johnson, 1988). Histidine-tagged Id proteins produced in the pRSET-B vector were purified on nickel-charged chelating agarose as recommended by the supplier (Invitrogen).

Electrophoretic mobility shift assays

Unlabelled proteins were synthesized by coupled transcription and translation of plasmid DNA using the TNT expression system (Promega) with either T7 or SP6 RNA polymerase. Samples (5 µl) of the different translation reactions were mixed and used for DNA-binding assays. Whole-cell extracts were prepared from TIG-3 cells according to published procedures (Andrews and Faller, 1991).

An E-box consensus sequence (CANNTG) from the muscle creatine kinase gene enhancer was used in all DNA-binding reactions. Two complementary oligonucleotides, 5'-GGATCCCCCAACACCTGCT-

GCCTGA-3' and 5'-TCAGGCAGCAGGTGTTGGGGGGAT-3', were annealed and labelled with [α -³²P]dCTP using the Klenow fragment of *E. coli* DNA polymerase. Two equivalent oligonucleotides containing a mutated E-box sequence, i.e. 5'-GGATCCCCCAAACTGGTCTG-CCTGA-3', were used as competitors in some experiments. DNA-binding reactions were done in a total volume of 20 µl containing 20 mM HEPES pH 7.6, 50 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 pre-incubated without the labelled probe, at room temperature for 10 min, and for a further 15 min at room temperature after addition of the labelled probe. The binding reactions were then subjected to electrophoresis in a 6% polyacrylamide gel in 25 mM Tris base, 25 mM boric acid and 0.5 mM EDTA (0.5× TBE) at 150 V for 2 h at room temperature. The gels were dried and the labelled complexes detected by autoradiography.

In vitro kinase assays

Sf9 insect cells were co-infected with the appropriate recombinant baculoviruses and whole-cell extracts were prepared 48 h post-infection as described by Kato *et al.* (1993). Phosphorylation reactions (final volume 40 µl) were performed in 10 mM HEPES pH 7.8, 1 mM MgCl₂, 1 mM dithiothreitol, 100 mM ATP and 5 µCi [γ -³²P]ATP and incubated with 1 µg of GST-Rb, GST-Id1 or GST-Id2 bound to glutathione-Sepharose beads. After 15 min at 37°C, the reactions were terminated by the addition of 1 ml of ice-cold buffer (NETN) containing 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA and 0.5% NP-40. The glutathione-Sepharose beads were recovered by centrifugation, washed several times in NETN, boiled and analysed by SDS-PAGE in 12% gel. In some experiments, the Sf9 cell lysates were preincubated for 10 min at 30°C with 20 ng of histidine-tagged p21^{CDKN1} protein.

Immunoblot analysis

Total cell lysates were fractionated by SDS-PAGE in a 12% gel according to standard protocols, and proteins were transferred to nitrocellulose filters as described previously (Bates *et al.*, 1994; Parry *et al.*, 1995). Id2 and cyclin A were detected with the SC-489 rabbit polyclonal antibody (Santa Cruz) or with the E23 mouse monoclonal antibody, respectively. Each antibody was used at 1:500 dilution in blocking buffer [4% milk powder and 0.2% Tween-20 in phosphate-buffered saline (PBS)] at room temperature for 1 h and immune complexes were detected by enhanced chemiluminescence (ECL) as described by the manufacturer (Amersham).

Phosphate labelling and immunoprecipitation

Asynchronously growing TIG-3 cells were made quiescent by placing them in medium containing 0.2% fetal bovine serum for 4 days. Serum was then added to 20% (v/v) and at 2, 6, 10, 14, 16 and 22 h after serum addition, the cells were incubated with 0.5 mCi/ml of ³²P-labelled orthophosphate for a further 2 h. The ³²P-labelled cells were washed with ice-cold PBS and lysates prepared in buffer (Ab buffer) containing 20 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.5% NP-40, 0.5% SDS, 0.5% deoxycholate, 1 mM EDTA and 100 µg of aprotinin/ml. Lysates were centrifuged at 15 000 r.p.m. for 30 min to remove debris and cleared by addition of protein A/G beads (Pierce) for 1 h at 4°C. Immunoprecipitations were performed with anti-Id2 antibody (SC-489) for 1 h at 4°C and the immune complexes recovered on protein A/G beads and washed four times with Ab buffer. The precipitated proteins were resuspended in sample buffer, boiled for 2 min and fractionated by SDS-PAGE on a 12% gel.

Two-dimensional phosphopeptide mapping

³²P-Labelled Id2 protein, generated either by phosphorylation *in vitro* or by metabolic labelling, was purified by SDS-PAGE in a 12% gel and transferred onto a PVDF membrane. The membrane was exposed to X-ray film to locate the Id2-specific bands. The relevant areas of the membrane were excised and subjected to trypsin digestion as described by Boyle *et al.* (1991). The resultant peptides were separated on cellulose thin-layer plates by electrophoresis at pH 1.9 (10 min at 0.5 kV and 40 min at 1 kV) in the first dimension, with subsequent ascending chromatography in the second dimension. The phosphopeptides were detected by autoradiography using Kodak X-AR 5 film at -80°C.

Acknowledgements

We thank Drs C.Murre, X.-H.Sun and A.Fujisawa-Sehara for the E12, E47 and MyoD cDNA clones, Drs E.Lees and T.Hunt for the cyclin E

and cyclin A antibodies, and members of the late H. Weintraub's laboratory for providing antiserum against MyoD. We are also indebted to Drs J. Campisi, F. Sablitzky and G. Spohr for useful suggestions and to R. Deed and J. Norton for sharing unpublished data. Richard Treisman, Nic Jones, David Ish Horowitz and Alison Sinclair provided helpful comments on the manuscript.

References

- Andrews, N.C. and Faller, D.V. (1991) A rapid micropreparation technique for extraction of DNA-binding proteins from small numbers of mammalian cells. *Nucleic Acids Res.*, **19**, 2499.
- Barone, M.V., Pepperkok, R., Peverali, F.A. and Philipson, L. (1994) Id proteins control growth induction in mammalian cells. *Proc. Natl Acad. Sci. USA*, **91**, 4985–4988.
- Bates, S., Bonetta, L., MacAllan, D., Parry, D., Holder, A., Dickson, C. and Peters, G. (1994) CDK6 (PLSTIRE) and CDK4 (PSK-J3) are a distinct subset of the cyclin-dependent kinases that associate with cyclin D1. *Oncogene*, **9**, 71–79.
- Begley, C.G., Aplan, P.D., Denning, S., Haynes, B.F., Waldmann, T.A. and Kirsch, I.R. (1989) The gene SCL is expressed during early hematopoiesis and encodes a differentiation-related DNA binding motif. *Proc. Natl Acad. Sci. USA*, **86**, 10128–10132.
- Beijersbergen, R.L., Carlee, L., Kerkhoven, R.M. and Bernards, R. (1995) Regulation of the retinoblastoma protein-related p107 by G₁ cyclin complexes. *Genes Dev.*, **9**, 1340–1353.
- Benezra, R., Davis, R.L., Lockshon, D., Turner, L. and Weintraub, H. (1990) The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell*, **61**, 49–59.
- Biggs, J., Murphy, E. and Israel, M. (1992) A human Id-like helix-loop-helix protein expressed during early development. *Proc. Natl Acad. Sci. USA*, **89**, 1512–1516.
- Boyle, W.J., van der Geer, P. and Hunter, T. (1991) Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.*, **201**, 110–149.
- Chen, Q. et al. (1990) The *tal* gene undergoes chromosome translocation in T cell leukemia and potentially encodes a helix-loop-helix protein. *EMBO J.*, **9**, 415–424.
- Christy, B.A., Sanders, L.K., Lau, L.F., Copeland, N.G., Jenkins, N.A. and Nathans, D. (1991) An Id-related helix-loop-helix protein encoded by a growth factor-inducible gene. *Proc. Natl Acad. Sci. USA*, **88**, 1815–1819.
- Crescenzi, M., Fleming, T.P., Lassar, A.B. and Weintraub, H. (1990) MyoD induces growth arrest independent of differentiation in normal and transformed cells. *Proc. Natl Acad. Sci. USA*, **87**, 8442–8446.
- Davis, R.L., Cheng, P.F., Lassar, A.B. and Weintraub, H. (1990) The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. *Cell*, **60**, 773–776.
- Deed, R.W., Bianchi, S.M., Atherton, G.T., Johnston, D., Santibanez-Koref, M., Murphy, J.J. and Norton, J.D. (1993) An immediate early human gene encodes an Id-like helix-loop-helix protein and is regulated by protein kinase C activation in diverse cell types. *Oncogene*, **8**, 599–607.
- Desprez, P.-Y., Hara, E., Bissell, M.J. and Campisi, J. (1995) Suppression of mammary epithelial cell differentiation by the helix-loop-helix protein Id1. *Mol. Cell. Biol.*, **15**, 3398–3404.
- Dowdy, S.F., Hinds, P.W., Louie, K., Reed, S.I., Arnold, A. and Weinberg, R.A. (1993) Physical interaction of the retinoblastoma protein with human D cyclins. *Cell*, **73**, 499–511.
- Dulic, V., Lees, E. and Reed, S.I. (1992) Association of human cyclin E with a periodic G₁-S phase protein kinase. *Science*, **257**, 1958–1961.
- Einarson, M.B. and Chao, M.V. (1995) Regulation of Id1 and its association with basic helix-loop-helix proteins during nerve growth factor-induced differentiation of PC12 cells. *Mol. Cell. Biol.*, **15**, 4175–4183.
- Ellenberger, T., Fass, D., Arnaud, M. and Harrison, S.C. (1994) Crystal structure of transcription factor E47: E-box recognition by a basic region helix-loop-helix dimer. *Genes Dev.*, **8**, 970–980.
- Ellis, H.M., Spann, D.R. and Posakony, J.W. (1990) Extramacrochaetae, a negative regulator of sensory organ development in *Drosophila*, defines a new class of helix-loop-helix proteins. *Cell*, **61**, 27–38.
- Ellmeier, W., Aguzzi, A., Kleiner, E., Kurzbauer, R. and Weith, A. (1992) A mutually exclusive expression of a helix-loop-helix gene and N-myc in human neuroblastomas and in normal development. *EMBO J.*, **11**, 2563–2571.
- Ephrussi, A., Church, G.M., Tonegawa, S. and Gilbert, W. (1985) B lineage-specific interactions of an immunoglobulin enhancer with cellular factors *in vivo*. *Science*, **227**, 134–140.
- Ewen, M.E., Sluss, H.K., Sherr, C.J., Matsushime, H., Kato, J. and Livingston, D.M. (1993) Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell*, **73**, 487–497.
- Garrell, J. and Modolell, J. (1990) The *Drosophila* extramacrochaetae locus, an antagonist of proneural genes that, like these genes, encode a helix-loop-helix protein. *Cell*, **61**, 39–48.
- Gu, W., Schneider, J.W., Condorelli, G., Kaushal, S., Mahdavi, V. and Nadal-Ginard, B. (1993) Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. *Cell*, **72**, 309–324.
- Hara, E., Kato, T., Nakada, S., Sekiya, S. and Oda, K. (1991) Subtractive cDNA cloning using oligo(dT)30-latex and PCR: isolation of cDNA clones specific to undifferentiated human embryonal carcinoma cells. *Nucleic Acids Res.*, **19**, 7097–7104.
- Hara, E., Yamaguchi, T., Nojima, H., Ide, T., Campisi, J., Okayama, H. and Oda, K. (1994) Id-related genes encoding helix-loop-helix proteins are required for G₁ progression and are repressed in senescent human fibroblasts. *J. Biol. Chem.*, **269**, 2139–2145.
- Hatakeyama, M., Brill, J.A., Fink, G.R. and Weinberg, R.A. (1994) Collaboration of G₁ cyclins in the functional inactivation of the retinoblastoma protein. *Genes Dev.*, **8**, 1759–1771.
- Henthorn, P., Kiledjian, M. and Kadesch, T. (1990) Two distinct transcription factors that bind the immunoglobulin enhancer μ E5/ κ E2 motif. *Science*, **247**, 467–470.
- Hu, J.-S., Olson, E.N. and Kingston, R.E. (1992) HEB, a helix-loop-helix protein related to E2A and ITF2 that can modulate the DNA-binding ability of myogenic regulatory factors. *Mol. Cell. Biol.*, **12**, 1031–1042.
- Iavarone, A., Garg, P., Lasorella, A., Hsu, J. and Israel, M.A. (1994) The helix-loop-helix protein Id2 enhances cell proliferation and binds to the retinoblastoma protein. *Genes Dev.*, **8**, 1270–1284.
- Jan, Y.N. and Jan, L.Y. (1993) HLH proteins, fly neurogenesis, and vertebrate myogenesis. *Cell*, **75**, 827–830.
- Jen, Y., Weintraub, H. and Benezra, R. (1992) Overexpression of Id protein inhibits the muscle differentiation program: *in vivo* association of Id with E2A proteins. *Genes Dev.*, **6**, 1466–1479.
- Johnson, J.E., Birren, S.J. and Anderson, D.J. (1990) Two rat homologues of *Drosophila* achaete-scute specifically expressed in neuronal precursors. *Nature*, **346**, 858–861.
- Johnson, J.E., Birren, S.J., Saito, T. and Anderson, D.J. (1992) DNA binding and transcriptional regulatory activity of mammalian achaete-scute homologous (MASH) protein revealed by interaction with a muscle-specific enhancer. *Proc. Natl Acad. Sci. USA*, **89**, 3596–3600.
- Kato, J., Matsushime, H., Hiebert, S.W., Ewen, M.E. and Sherr, C.J. (1993) Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev.*, **7**, 331–342.
- Kiledjian, M., Su, L.-K. and Kadesch, T. (1988) Identification and characterisation of two functional domains within the murine heavy-chain enhancer. *Mol. Cell. Biol.*, **8**, 145–152.
- Klamt, C., Knust, E., Tietze, K. and Campos-Ortega, J.A. (1989) Closely related transcripts encoded by the neurogenic gene complex enhancer of split of *Drosophila melanogaster*. *EMBO J.*, **8**, 203–210.
- Koff, A. et al. (1992) Formation and activation of a cyclin E-cdk2 complex during the G₁ phase of the human cell cycle. *Science*, **257**, 1689–1694.
- Kreider, B., Benezra, R., Rovera, G. and Kadesch, T. (1992) Inhibition of myeloid differentiation by the helix-loop-helix protein Id. *Science*, **255**, 1700–1702.
- Lam, E.W.-F. and La Thangue, N.B. (1994) DP and E2F proteins: coordinating transcription with cell cycle regulation. *Curr. Opin. Cell Biol.*, **6**, 859–866.
- Lasorella, A., Iavarone, A. and Israel, M.A. (1996) Id2 specifically alters regulation of the cell cycle by tumor suppressor proteins. *Mol. Cell. Biol.*, **16**, 2570–2578.
- Lassar, A.B., Buskin, J.N., Lockshon, D., Davis, R.L., Apone, S., Hauschka, S.D. and Weintraub, H. (1989) MyoD is a sequence-specific DNA protein requiring a region of *myc* homology to bind to the muscle creatine kinase enhancer. *Cell*, **58**, 823–831.
- Lassar, A.B., Davis, R.L., Wright, W.E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D. and Weintraub, H. (1991) Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins *in vivo*. *Cell*, **66**, 305–315.

- Lee, J.E., Hollenberg, S.M., Snider, L., Turner, D.L., Lipnick, N. and Weintraub, H. (1995) Conversion of *Xenopus* ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science*, **268**, 836–844.
- Le Jossic, C., Ilyin, G.P., Loyer, P., Glaize, D. and Cariou, S. (1994) Expression of helix-loop-helix factor Id-1 is dependent on the hepatocyte proliferation and differentiation status in rat liver and in primary culture. *Cancer Res.*, **54**, 6065–6068.
- Lister, J., Forrester, W.C. and Baron, M.H. (1995) Inhibition of an erythroid differentiation switch by the helix-loop-helix protein Id1. *J. Biol. Chem.*, **270**, 17939–17946.
- Ma, P.C., Rould, M.A., Weintraub, H. and Pabo, C.O. (1994) Crystal structure of MyoD bHLH domain–DNA complex: perspectives on DNA recognition and implications for transcriptional activation. *Cell*, **77**, 451–459.
- Matsushime, H., Quelle, D.E., Shurtleff, S.A., Shibuya, M., Sherr, C.J. and Kato, J.-Y. (1994) D-type cyclin-dependent kinase activity in mammalian cells. *Mol. Cell. Biol.*, **14**, 2066–2076.
- Mellentin, J.D., Smith, S.D. and Cleary, M.L. (1989) *lxl-1*, a novel gene altered by chromosomal translocation in T cell leukemia codes for a protein with a helix-loop-helix DNA binding motif. *Cell*, **58**, 77–83.
- Meyerson, M. and Harlow, E. (1994) Identification of G₁ kinase activity for cdk6, a novel cyclin D partner. *Mol. Cell. Biol.*, **14**, 2077–2086.
- Murre, C., McCaw, P.S. and Baltimore, D. (1989a) A new DNA binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, MyoD, and myc proteins. *Cell*, **56**, 777–783.
- Murre, C. *et al.* (1989b) Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell*, **58**, 537–544.
- Nagata, Y., Shoji, W., Obinata, M. and Todokoro, K. (1995) Phosphorylation of helix-loop-helix proteins ID1, ID2 and ID3. *Biochem. Biophys. Res. Commun.*, **207**, 916–926.
- Naya, F.J., Stellrecht, C.M.M. and Tsai, M.-J. (1995) Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. *Genes Dev.*, **8**, 1009–1019.
- Ohashi, M. *et al.* (1980) A new human diploid cell strain, TIG-1, for the research on cellular ageing. *Exp. Gerontol.*, **15**, 121–133.
- Ohtsubo, M., Theodoras, A.M., Schumacher, J., Roberts, J.M. and Pagano, M. (1995) Human cyclin E, a nuclear protein essential for the G₁-to-S phase transition. *Mol. Cell. Biol.*, **15**, 2612–2624.
- Olson, E.N. (1990) The MyoD family, a paradigm for development? *Genes Dev.*, **4**, 1454–1461.
- Olson, E.N. and Klein, W.H. (1994) bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. *Genes Dev.*, **8**, 1–8.
- Pagliuca, A., Cannada, P., Bartoli, C., Saccone, S., Della Valle, G. and Lania, L. (1995) Molecular cloning of ID4, a novel dominant negative helix-loop-helix human gene on chromosome 6p21.3–p22. *Genomics*, **27**, 200–203.
- Parry, D., Bates, S., Mann, D.J. and Peters, G. (1995) Lack of cyclin D–cdk complexes in Rb-negative cells correlates with high levels of p16^{INK4MTS1} tumor suppressor gene product. *EMBO J.*, **14**, 503–511.
- Peters, G. (1994) The D-type cyclins and their role in tumorigenesis. *J. Cell Sci. Suppl.*, **18**, 89–96.
- Peverali, F.A., Ramqvist, T., Saffrich, R., Pepperkok, R., Barone, M.V. and Philipson, L. (1994) Regulation of G₁ progression by E2A and Id helix-loop-helix proteins. *EMBO J.*, **13**, 4291–4301.
- Resnitzky, D. and Reed, S.I. (1995) Different roles for cyclins D1 and E in regulation of the G₁-to-S transition. *Mol. Cell. Biol.*, **15**, 3463–3469.
- Riechmann, V., Van Cruchten, I. and Sablitzky, F. (1994) The expression pattern of Id4, a novel dominant negative helix-loop-helix protein, is distinct from Id1, Id2 and Id3. *Nucleic Acids Res.*, **22**, 749–755.
- Shen, C.-P. and Kadesch, T. (1995) B-cell specific DNA binding by an E47 homodimer. *Mol. Cell. Biol.*, **15**, 4518–4524.
- Sherr, C.J. (1994) G₁ phase progression: cycling on cue. *Cell*, **79**, 551–555.
- Shoji, W., Yamamoto, T. and Obinata, M. (1994) The helix-loop-helix protein Id inhibits differentiation of murine erythroleukemia cells. *J. Biol. Chem.*, **269**, 5078–5084.
- Shoji, W., Inoue, T., Yamamoto, T. and Obinata, M. (1995) MIDA1, a protein associated with Id, regulates cell growth. *J. Biol. Chem.*, **270**, 24818–24825.
- Skerjanc, I.S., Truong, J., Filion, P. and McBurney, M.W. (1996) A splice variant of the ITF-2 transcript encodes a transcription factor that inhibits MyoD activity. *J. Biol. Chem.*, **271**, 3555–3561.
- Smith, D.B. and Johnson, K.S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene*, **67**, 31–40.
- Sorrentino, V., Pepperkok, R., Davis, R.L., Ansorge, W. and Philipson, L. (1990) Cell proliferation inhibited by *MyoD1* independently of myogenic differentiation. *Nature*, **345**, 813–815.
- Srivastava, D., Cserjesi, P. and Olson, E.N. (1995) A subclass of bHLH proteins required for cardiac morphogenesis. *Science*, **270**, 1995–1999.
- Staudinger, J., Perry, M., Elledge, S.J. and Olson, E.N. (1993) Interactions among vertebrate helix-loop-helix proteins in yeast using the two-hybrid system. *J. Biol. Chem.*, **268**, 4608–4611.
- Sun, X.-H. (1994) Constitutive expression of the *Id1* gene impairs mouse B cell development. *Cell*, **79**, 893–900.
- Sun, X.-H., Copeland, N.G., Jenkins, N.A. and Baltimore, D. (1991) Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. *Mol. Cell. Biol.*, **11**, 5603–5611.
- Tietze, K., Oellers, N. and Knust, E. (1992) Enhancer of split D, a dominant mutation of *Drosophila*, and its use in the study of functional domains of a helix-loop-helix protein. *Proc. Natl Acad. Sci. USA*, **89**, 203–210.
- Weinberg, R.A. (1995) The retinoblastoma protein and cell cycle control. *Cell*, **81**, 323–330.
- Weintraub, H. (1993) The MyoD family and myogenesis: redundancy, networks, and thresholds. *Cell*, **75**, 1241–1244.
- Weintraub, H. *et al.* (1991) The myoD gene family: nodal point during specification of the muscle cell lineage. *Science*, **251**, 761–766.
- Wilson, R. and Mohun, T. (1995) Xid, a dominant negative regulator of bHLH function in early *Xenopus* embryos. *Mech. Dev.*, **49**, 211–222.
- Zhang, H., Reynaud, S., Kloc, M., Etkin, L.D. and Spohr, G. (1995) ID gene activity during *Xenopus* embryogenesis. *Mech. Dev.*, **50**, 119–130.
- Zhang, Y., Babin, J., Feldhaus, A.L., Singh, H., Sharp, P.A. and Bina, M. (1991) HTF4: a new human helix-loop-helix protein. *Nucleic Acids Res.*, **19**, 4555.

Received on August 27, 1996; revised on September 23, 1996