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E2A basic-helix–loop–helix transcription factors are negatively regulated by serum growth factors and by the Id3 protein

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ABSTRACT

Id3, a member of the Id multigene family of dominant negative helix–loop–helix transcription factors, is induced sharply in murine fibroblasts by serum growth factors. To identify relevant targets of Id3 activity, the yeast two–hybrid system was used to identify proteins that dimerize with Id3. Four murine cDNAs were identified in the screen, all of which encode helix–loop–helix proteins: E12, E47, ALF1 and Id4. Co-immunoprecipitation assays confirm that Id3 interacts with E12, E47 and two alternative splice products of ALF1 *in vitro.* Id3 disrupts DNA binding by these proteins *in vitro* and blocks transcriptional activation by these factors in cultured murine cells. Additionally, Id3 shows evidence of interacting with the related proteins E2-2 and MyoD, but not c-Myc. These results suggest that Id3 can function as a general negative regulator of the basic-helix–loop–helix family of transcription factors exemplified by the 'E' proteins and MyoD. Although it was previously suspected that E2A is constitutively expressed, our data indicate that E2A is induced in quiescent fibroblasts, by growth factor withdrawal but not by contact inhibition of cell proliferation. These observations extend the role of Id3 in the functional antagonism of E2A-class transcription factors, and suggest that E2A proteins may mediate growth inhibition.

INTRODUCTION

The investigation of genes induced by mitogenic growth factors has led to the identification of many genes that mediate the growth response. Most of the genes transcribed in the first 1–3 h after growth factor stimulation, called the immediate early response genes, encode transcription factors. These factors directly regulate subsequent programs of gene activation important to cell cycle progression (1–3). Many of these growth-promoting transcription factors are proto-oncogenes whose mutational activation contributes to neoplastic transformation (4,5).

Id3 is one representative of the immediate early response class of genes in fibroblasts. The murine cDNA, originally called clone 462, was identified as an anonymous transcript induced in serum-starved BALB/c 3T3 murine fibroblasts by stimulation with fetal bovine serum (6). The mRNA is also induced by platelet-derived growth factor, phorbol esters and calcium ionophore. It was observed that clone 462 encodes a protein bearing a helix–loop–helix motif, a dimerization interface common to a family of transcription factors, and it was renamed HLH462 (7,8). Subsequently, two groups have identified human homologs. Heir1 has been identified as a CpG island gene in a region of chromosome 1p deleted in human neuroblastoma (9). The human homolog also has been isolated as a phorbol ester-inducible transcript, called HLH 1R21 (10). After the identification of Id1 and Id2, it was recognized that HLH462 constitutes an additional member of this multigene family, and the name Id3 was proposed (11). An additional family member, Id4, has been recognized (12).

The Id proteins share with each other considerable amino acid sequence homology in the HLH domain. However, outside this domain of ~40 amino acids, their sequences diverge sharply. They form a subclass of a larger HLH family that includes basic-helix–loop–helix (bHLH) transcriptional activators (13–17). Such proteins dimerize via the HLH to bind to specific DNA sequences recognized by the paired basic regions (18–20). Since Id proteins lack a basic motif, their dimerization with bHLH proteins inhibits DNA binding *in vitro*, and this results in loss of transcriptional activation by the bHLH factor *in vivo*. Two classes of bHLH proteins have been distinguished. The first class, termed E proteins, includes nearly ubiquitously expressed bHLH proteins that can bind DNA as either homodimers or heterodimers. This class is exemplified by E12 and E47, alternative splice products of the E2A gene, as well as the closely related genes E2-2 and ALF1/HEB (13,21–24). The second class includes bHLH proteins with a restricted expression pattern, for example MyoD in skeletal muscle (25), TAL1/SCL in hematopoietic cells (26,27) and MASH1 in neural tissue (28). Proteins in this second class appear to require heterodimerization with an E protein to assume functional activity (14,29,30).

The function of Id1 on bHLH proteins has been well investigated, but much less is known about the function of the other Id proteins. In general, the Id genes are highly expressed during embryogenesis and in undifferentiated cells, decreasing during tissue differentiation (8,11,12,31–36). Overexpression of Id1 inhibits tissue-specific

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gene transcription in many cultured cell types, including skeletal muscle, cardiac muscle, myeloid cells, lymphocytes, pancreatic β cells and osteoblasts (37–43). In addition, enforced Id1 expression actually blocks differentiation in skeletal muscle, myeloid and murine erythroleukemia cells (39,44,45). Finally, enforced expression of Id1 targeted to B lymphocytes of transgenic mice interferes with normal B lymphocyte maturation (46). Thus Id proteins appear to regulate cell fate in a variety of tissue types.

The expression pattern of Id3 is even more extensive than that of Id1. Murine Id3 mRNA is readily detectable in brain, lung, thymus, intestine, muscle, spleen and liver (8). Expression of the human homolog also has been found in pancreas, kidney, placenta, adrenal medulla and heart (9,10). The widespread expression of Id3 mRNA suggests that the Id3 protein may serve a generalized function important to many cell types.

Because Id expression follows a pattern similar to other immediate early response genes, it is likely that Id proteins also play a regulatory role in cellular proliferation. Id1, Id2 and Id3 transcripts are abundant in proliferating fibroblasts, but are undetectable in quiescent or senescent fibroblasts (8,47,48). The Id3 gene is highly responsive to diverse mitogenic pathways, including growth factors, protein kinase C, adenylate cyclase and calcium flux (8). As discussed above, Id genes are highly expressed in proliferating, undifferentiated cells, declining with differentiation and loss of proliferative potential. Thus, Id gene expression is consistently linked with cell proliferation. In further support of this model, antisense experiments demonstrate that the expression of Id proteins is critical for cell cycle progression into S phase (47,48). A direct role for Id2 in antagonizing the growth suppressive function of the retinoblastoma gene product has been proposed (49).

Although it is likely that Id3 might share known targets with the other Id family members, Id targets have never been sought systematically. According to the current model of Id-class protein function, we hypothesized that Id3 exerts its function through protein–protein interaction via its HLH domain. It is likely that such a target would exist in the cell prior to the onset of growth factor-induced Id3 gene transcription and translation. We chose the two-hybrid system of expression cloning to identify potential physiological targets, followed by evaluation of other related proteins for similar interactions with Id3. The following data characterize Id3 as a general negative regulator of E2A-class bHLH transcription factors. In addition, we find that E2A gene expression is upregulated during G_0 arrest by withdrawal of serum growth factors and downregulated during G_1 re-entry by growth factor stimulation.

MATERIALS AND METHODS

cDNA library construction and two-hybrid screening

BALB/c 3T3 murine fibroblasts were grown as described (7). After 3 days in low serum medium as described, mRNA was harvested and purified by oligo-dT affinity (Fast-Track Kit, Invitrogen). Ten micrograms of mRNA were used to generate and clone cDNA (Superscript plasmid cloning system, Gibco-BRL), which was ligated directionally between the *Sal*I and *Not*I sites of pPC86 (50). This library was screened in yeast bearing a gene encoding the yeast GAL4 DNA-binding domain (amino acids 1–147) fused to murine Id3 amino acids 33–119, inserted by homologous recombination into the LEU2 locus, and verified by Southern blotting. The two-hybrid plasmid vectors, initial yeast strains and screening methods have been previously described (50). Library plasmids encoding Id3 binding proteins were characterized by partial DNA sequencing (Sequenase kit, USB Biochemical) or restriction mapping of characteristic *Pvu*II sites in E2A cDNA clones.

Plasmid constructs

Plasmids were gifts as following: pBSKSII462-3 murine Id3 cDNA (8), pBS-HLH31-5 murine Id4 cDNA, murine c-Fos and c-Jun expression vectors, pColl-CAT collagenase promoter-CAT reporter (51), pPC86 and pPC62 two-hybrid plasmids and pPC67 mouse 14.5d embryo cDNA library (50), D. Nathans (Johns Hopkins University); pEMCIIs murine MyoD cDNA (52), pE:Id murine Id1 cDNA (37) and pTK-MCK-4R-CAT reporter (53), H. Weintraub (Fred Hutchinson Cancer Center); partial and full length human E12 and E47 plasmids (18), D. Baltimore (Rockefeller University); pCDM8-HEB1A and pBSATG-HEBr (24), R. Kingston (Harvard Medical School); p44tot ALF1A and p11tot ALF1B murine cDNA (23), P. Jørgensen (University of Aarhus, Denmark); pT7βE2-2 cDNA and $p(5+2)_4$CAT reporter (21), T. Kadesch (University of Pennsylvania), pGAL4-MyoD (54), G. Tomaselli, (Johns Hopkins University); pSP65Myc, C. Dang (Johns Hopkins University), pYN3214 lacZ expression vector, pcDEBΔ expression vector, and pcDEBΔ:Neo expression vector, Y. Nakabeppu (Kyushu University, Fukuoka, Japan). Inserts from the Id3, E12 and GAL4-MyoD plasmids were subcloned in frame downstream of the GAL4 transcriptional activation domain (TAD) in the pPC86 plasmid and downstream of the GAL4 DNA binding domain (DBD) in the pPC62 plasmid. Inserts encoding the full length E12, E47, ALF1A and ALF1B were subcloned into the pcDEBΔ:Neo mammalian expression vector. Inserts encoding full length Id3 and mutant Id3R72P were subcloned into the pcDEBΔ mammalian expression vector.

Co-immunoprecipitation assays

Using the above plasmids as templates, supercoiled or linearized plasmid DNA was transcribed and translated *in vitro* in the presence of [^{35}S]methionine (DuPont NEN) using the TnT kit (Promega). Id3, Id3R72P, HEBr, E47, MyoD and Id1 were transcribed with T3 RNA polymerase. ALF1A, ALF1B, E12, Id4 and E2-2 were transcribed with T7 RNA polymerase. SP6 RNA polymerase was used to transcribe c-Myc. Aliquots were run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and activity was quantitated by phosphorimager (Molecular Dynamics) to allow usage of approximately equal counts/min of radiolabeled proteins. Aliquots of each radiolabeled protein were mixed with radiolabeled Id3 or buffer control, and then incubated at 37°C for 20 min. Precipitations were performed in 150 µl immunoprecipitation buffer as described (55). The reactions were precleared with 10 µl of rabbit serum and protein A–Sepharose, then precipitated with 10 µl of rabbit Id3 antiserum (gift of D. Nathans) and protein A–Sepharose. The precipitated proteins were boiled in 1% SDS, 50 mM Tris–HCl pH 6.8, 100 mM DTT, and fractionated on 12% SDS–PAGE gels. The gels were treated with EnHance (DuPont NEN), dried and autoradiographed at –85°C.

Electrophoretic mobility shift assays (EMSA)

Proteins were translated *in vitro* without isotopic label in parallel with the above reactions. Efficiency of the reactions was

monitored by radioactive incorporation in the isotopic translation. The oligonucleotide probe was labeled with [α-^{32}P]dATP (DuPont NEN) using Klenow fragment. Dilutions of translation reactions equivalent to 0.15–1 µl were incubated with 10^5 c.p.m. of probe in 10 µl of 12% glycerol, 12 mM HEPES–NaOH (pH 7.9), 4 mM Tris–HCl, 60 mM KCl, 1 mM EDTA and 1 mM DTT with 1 µg poly(dI–dC) at 25°C for 15 min, and then loaded on a 5% polyacrylamide gel (60:1 bis) Tris-borate–EDTA and run at 200 V for ~3 h. The gel was dried and autoradiographed at –85°C using an intensifying screen. Id3 blocking reactions received 1–8 µl of *in vitro* translated Id3. All such reactions received unprogrammed reticulocyte lysate to keep the total amount of lysate constant in each reaction, to control for background DNA binding activities.

Id3 mutagenesis

A mutational oligonucleotide primer, TGGAAATCCTGCAGCCT-GTCA, was used in conjunction with a downstream antisense primer, GGTCAGTGGCAAAACCTCCTC, and the Id3 cDNA plasmid template to amplify an internal 164 nt fragment. This was cleaved with *Pst*I and *Sac*I and inserted into similarly cleaved pBSKSII462-3. The desired mutation was confirmed by dideoxy-nucleotide sequencing of both strands. The full length insert carrying the R72P mutation was subcloned into the pcDEBΔ expression vector. Primers were produced on an Applied Biosystems 380B oligonucleotide synthesizer as recommended by the manufacturer.

Transcriptional activation assays

All transient transfections were carried out by calcium phosphate precipitation in NIH-3T3 murine fibroblasts as described (55). Each transfection included 5 µg $p(5+2)_4$CAT reporter plasmid and 7.5 µg pYN3214 lacZ expression vector. Each transfection included either 10 µg of ALF1A or ALF1B expression vector, 5 µg E12 expression vector or 0.5 µg E47 expression vector. Each transfection also included either: (i) 20 µg pcDEBΔ empty vector, (ii) 5 µg Id3 expression vector plus 15 µg pcDEBΔ, (iii) 20 µg Id3 or (iv) 20 µg Id3R72P mutant. The negative control transfections included 5 µg c-Fos and 5 µg c-Jun expression vectors, 5 µg pColl-CAT, and the indicated amounts of Id3 or pcDEBΔ plasmids. β-galactosidase assays were performed as described (56), and the results were used to normalize CAT assays, performed as described (55). Quantitations were performed by phophorimager or analysis of scanned autoradiograms on Image Quant software (Molecular Dynamics).

Northern blots

Total RNA was extracted from serum starved and serum stimulated BALB/c 3T3 fibroblasts as described (8). Aliquots of each sample (10–20 µg) were electrophoresed on a 1.2% agarose–formaldehyde gel, and transferred to nitrocellulose membranes. Probe fragments were prepared from the library murine cDNA isolates: the 2.0 kb ALF1 and the 0.9 kb E47 *Sal*I–*Not*I inserts. The murine Id3 0.9 kb insert was prepared from pBSKSII462-3. The human glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA fragment was cleaved from pGD-P-25A, obtained from the American Type Culture Collection. The fragments were labeled with [α-^{32}P]dCTP by the random primer method (Gibco-BRL) and purified on NICK columns (Pharmacia). Blots were hybridized as described (8) and washed in 2× SSC, 0.2% SDS at 42°C for 1.5 h, followed by 0.4× SSC, 0.1% SDS at 50°C for 1.5 h, followed by autoradiography with intensifying screens at –85°C.

Anti-E2A antibody production and immunoblot analysis

The following PCR primers were used to generate a 281 base pair fragment of murine E2A coding sequence, corresponding to amino acids 444–529 of human E2A: CGCGGATCCTGGGCGGG-C-GGCATGCC and CCCGGATCCGTTAACTGGTGCGCGTG-CGTGGGA. The fragment was subcloned into pDS-MCS (a gift of F. Rauscher, Wistar Institute), expressing a hexahistidine fusion protein in *Escherichia coli* that was purified as described (57). New Zealand white rabbits were immunized with the fusion protein (Hazelton Research Products). The resulting antiserum was affinity purified on a column of the fusion protein conjugated to activated CH Sepharose 4B (Pharmacia), eluted as described (58). BALB/c 3T3 fibroblasts were serum starved for 3 days, and the stimulated with 20% fetal bovine serum for various times. The cells were lysed in 1% SDS and heated to 100°C for 10 min. Protein concentration was determined by the BCA assay (Pierce). Twenty micrograms of whole cell extract per lane was fractionated on a 10% SDS–PAGE, and electroblotted to a nitrocellulose-nylon membrane. Nuclear extracts were prepared by incubating the cells on ice for 5 min in 10 mM Tris–HCl pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 0.5% Nonidet P-40, 0.7 µg/ml pepstatin A, 0.5 µg/ml leupeptin, 1 mM PMSF, followed by centrifugation for 5 min at 6000 *g*. The nuclear pellet was washed with the same buffer, centrifuged, then suspended in 1% SDS as above. One hundred micrograms of nuclear extract were loaded per lane. The blots were probed with a 1:1000 dilution of E2A antibody and developed by enhanced chemiluminescence as recommended by the manufacturer (Amersham).

RESULTS

The murine E12, E47, ALF1 and Id4 HLH proteins are identified in the two-hybrid system as potential Id3 targets

We hypothesized that induction of Id3 expression from very low background levels leads to its interaction with pre-existing protein factors. Since Id3 mRNA is induced rapidly after growth factor stimulation, it seemed likely that the Id3 targets would be expressed before growth factor stimulation. Using vectors previously described, we screened a cDNA library derived from whole mouse embryos with a construct expressing the GAL4 DNA binding domain (DBD) fused with nearly full length murine Id3 (amino acids 33–119). In order to specifically seek Id3 associated proteins existing before Id3 induction, we constructed a GAL4 transcriptional activation domain-fusion cDNA library from mRNA derived from serum starved, quiescent fibroblasts. This oligo-dT primed library contains 1.6×10^6 recombinants, of average size 1 kb and size range 0.6–3.4 kb (data not shown). Screening of the two libraries yielded a total of 74 clones. Nucleotide sequence analysis revealed three classes of cDNAs. Seventy-two isolates are murine homologs of the human E2A gene transcripts E12 and E47. Of 18 isolates selected for further analysis, 12 are identical to murine A1 (59) encoding E47 homologs, and four encode E12 homologs. An additional isolate is identical to murine ALF1, a cDNA derived from a distinct gene very closely related to E2A (23). A final isolate encodes Id4, an additional Id family member (12). Additionally, the protein products of these isolates all can associate with a minimal HLH domain from Id3 (amino acids 33–88) fused to the GAL4 DBD, but not with the GAL4 DBD alone (data not shown). Lastly, GAL4 DBD/Id3 was found to interact with a GAL4 TAD/MyoD

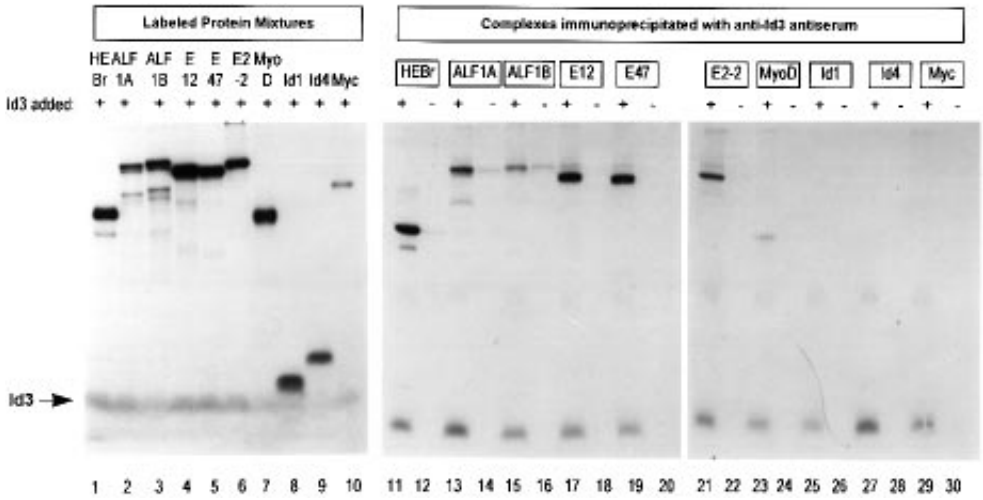

Figure 1. Id3 associates with bHLH proteins *in vitro*. Various radiolabeled bHLH proteins were produced by *in vitro* transcription and translation. These proteins were mixed with *in vitro* translated Id3, electrophoresed and autoradiographed (left panel). Duplicate reactions with and without added Id3 were immunoprecipitated with anti-Id3 antiserum. The precipitated complexes were electrophoresed and autoradiographed (center and right panels).

fusion in the two-hybrid system (data not shown). A high degree of specificity was observed in the screening; all isolates encoded HLH proteins, and none encoded nonspecifically interacting proteins. These results imply that E2A gene transcripts constitute the most abundant mRNAs encoding dimerization targets of Id3 protein in murine embryos and fibroblasts. Furthermore, the HLH motif provides a very high level of specificity in the yeast two-hybrid screen. We conclude that Id3 can interact with E12, E47, ALF1, Id4 and MyoD in yeast.

Id3 protein binds to E2A-class HLH proteins *in vitro*

To confirm the above results obtained with fusion proteins in yeast, further studies were conducted *in vitro*. Full length *in vitro* translated proteins were used in coimmunoprecipitation assays. The translation product of each of the library isolates was evaluated, along with several closely related HLH cDNAs: E12, E47, ALF1A, ALF1B (alternative splice products of the *alf1* gene), HEB (a truncated human homolog of ALF1), E2-2 (the product of another E2A multigene family member), MyoD, Id1 and Id4 (Fig. 1). c-Myc, a member of a structurally distinct bHLH-ZIP family, was chosen as a negative control. These family members possess a bHLH motif contiguous with a leucine zipper motif. Such bHLH-ZIP family members have never been shown to interact with E2A-class bHLH proteins (13,60). When the above proteins are mixed with Id3 protein, antiserum directed against Id3 efficiently coimmunoprecipitates E12, E47, ALF1A, ALF1B, HEB and E2-2 (for convenience, the products of this multigene family will be referred to here collectively as E proteins). MyoD and Id4 coimmunoprecipitate with Id3 less efficiently. Id1 and c-Myc show no detectable coimmunoprecipitation with Id3. These data suggest that Id3 protein will heterodimerize with E2A-class bHLH proteins, but not with some other HLH proteins and not with Myc-class bHLH-ZIP proteins.

Id3 blocks DNA binding by E2A-class bHLH proteins

To evaluate the functional consequences of heterodimerization with Id3, DNA binding analysis of dimers of bHLH proteins was performed using the electrophoretic mobility shift assay (EMSA). Although some bHLH proteins can bind to DNA as homodimers, some bind DNA only as heterodimers with certain proteins (14). Where feasible, the activity of Id3 was evaluated on both homo- and heterodimers of the E proteins. E47 and HEB homodimers readily bind to the muscle creatine kinase (MCK) enhancer site (Fig. 2). E12 homodimers bind only poorly to DNA, consistent with previously published data (61). Due to homodimerization of E2-2 translation products of heterogeneous length, the resulting DNA–protein complex is diffuse and barely visualized by EMSA. In each case, any observable binding was inhibited by the addition of Id3 protein (Fig. 2). *In vitro* translation of ALF1A and ALF1B yielded many different products, apparently due to internal initiation, and were unsuitable for EMSA analysis (data not shown). HEBr, the truncated human homolog of ALF1A, was used for EMSA analysis instead. In agreement with previous data, the following proteins were found to bind as heterodimers with MyoD to the MCK enhancer: E12, E47, HEBr and E2-2. Titration of Id3 protein into the binding reactions inhibited DNA binding by these heterodimers (Fig. 2). This indicates that the functional consequence of Id3 heterodimerization with an E protein is inhibition of DNA binding.

Id3 inhibits transcriptional activation by E proteins

To determine whether this inhibition of DNA binding resulted in loss of transcriptional activation by E proteins, transfection experiments were performed in cultured cells. E proteins have previously been demonstrated to activate transcription from the MCK and μ immunoglobulin heavy chain enhancers (21,24,62). By transient transfection of appropriate expression constructs with a CAT reporter construct, E12, E47, ALF1A and ALF1B are found to activate transcription through the MCK enhancer (data not shown). For further analysis, CAT constructs linked to the μE5+μE2 enhancer were chosen, because this yielded greater sensitivity than the MCK enhancer. Expression of any of these bHLH proteins yielded significant transcriptional activation of CAT through the μE5+μE2 enhancer. Titration of increasing amounts of Id3 expression plasmid resulted in progressive loss of

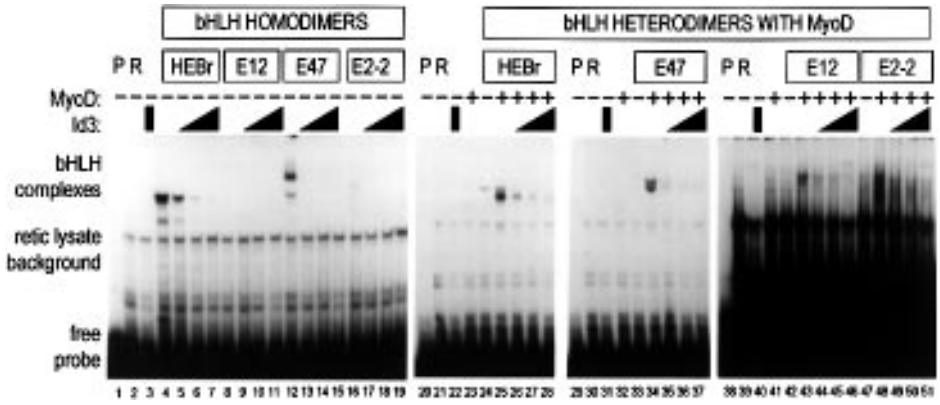

Figure 2. Id3 blocks DNA binding by bHLH proteins *in vitro*. Unlabeled bHLH and Id3 proteins were produced *in vitro* in parallel reactions to those from Figure 1. Minimal amounts of bHLH proteins were mixed with 0, 1, 2 or 4 µl of Id3 translation mix, and then with radiolabeled oligonucleotide containing the MCK enhancer, followed by fractionation by polyacrylamide gel electrophoresis and autoradiography. Reactions also contained 4, 3, 1 and 0µl of unprogrammed reticulocyte lysate as a control. Lanes are marked as follows: P, probe DNA without added proteins (lanes 1, 20, 29 and 38); R, unprogrammed reticulocyte lysate (lanes 2, 21, 30, 39); ■, 4 µl of Id3 translation mix (lanes 3, 22, 31 and 40). The wedge indicates 1, 2 and 4µl of Id3 added (lanes 5–7, 9–11, 13–15, 17–19, 26–28, 35–37, 44–46 and 49–51). The left panel shows the effect of increasing amounts of Id3 on DNA binding by homodimeric HEBr (lanes 4–7), E12 (lanes 8–11), E47 (lanes 12–15) and E2–2 (lanes 16–19). The remaining panels show the effect of increasing amounts of Id3 on DNA binding by heterodimeric complexes of MyoD with HEBr (lanes 24–28), E47 (lanes 33–37), E12 (lanes 42–46) and E2–2 (lanes 47–52). The binding reactions containing added MyoD are indicated (lanes 23, 25–28, 32, 34–37, 41, 43–46 and 48–51).

transcriptional activation (Fig. 3). To provide a negative control, a point mutant was designed with a helix-breaking proline substitution in the second helix of Id3. This mutant, referred to as Id3R72P, would not be predicted to dimerize with other proteins due to structural disruption of the HLH dimerization interface. As expected, this mutant was ineffective in inhibiting transcriptional activation by E12, E47, ALF1A and ALF1B (Fig. 3). In addition, this mutant protein failed to coimmunoprecipitate E47 *in vitro* (data not shown). As expected, Id3 was ineffective in inhibiting transcriptional activation of a collagenase promoter by c-Jun alone, or c-Fos with c-Jun, transcriptional activators not belonging to the bHLH class (Fig. 3). These data show that Id3 acts as a dominant negative regulator of transcriptional activation by E proteins.

E2A gene expression is regulated during cell cycle re-entry

We carried out northern blot analysis out in order to document the expression of the E2A and ALF1 genes in the murine fibroblast population from which they were isolated. Serum starved and serum stimulated BALB/c 3T3 cells express transcripts of sizes similar to those reported previously (23,59). The results confirm high level expression of E2A transcripts in quiescent cells. E2A transcripts are also detectable after serum stimulation, with marked downregulation seen at 2–4 h, rising again as the cells approach a second cell cycle (Fig. 4A). Id3 transcripts are induced at 1–2 h (Fig. 4A), as previously described (8).We also investigated whether Id3 is also induced around the time of S phase entry at ~12 h, as previously described for Id1 and Id2 (48). We did not detect a second peak of Id3 transcription, although a brief rise between the times of our sample collection cannot be excluded (Fig. 4B). The rise of E2A and Id3 transcripts at 16–24 h in Figure 4A is seen only when conditions are permissive for a second cell division (data not shown). Transcripts of ALF1 were detected at all time points at a very low level (data not shown). No clear changes in ALF1 expression were seen following serum stimulation.

Immunoblot analysis shows that E2A proteins accumulate in quiescent cells, falling with serum stimulation (Fig. 5A). These results confirm that E2A proteins are expressed in quiescent fibroblasts, constituting abundant, pre-existing potential targets for mitogen-induced Id3 protein. The level of E2A transcript and protein is regulated in association with proliferative status. Higher E2A mRNA levels are associated with cellular quiescence, consistent with the observation that enforced E2A expression causes growth arrest (63). We performed a second analysis of the expression of E2A to more closely evaluate the period of S phase entry ~12 h. No additional significant regulation of E2A proteins level was seen (Fig. 5B).

Although under normal conditions, E2A proteins disappear as the mRNA declines, the protein synthesis inhibitor cycloheximide inhibits the degradation of existing E2A proteins (Fig. 5C). This indicates that a serum-induced or labile protein factor is required for the turnover of E2A proteins. Finally, E2A expression is negatively regulated by serum growth factors, but not by contact inhibition of cell proliferation (Fig. 5D). Thus E2A induction coincides with withdrawal of serum, suggesting that E2A transcription factors may mediate cellular quiescence due to absence of growth factor.

DISCUSSION

Id and E proteins in cell proliferation

Data accumulating over the last several years increasingly point to a role of the HLH transcription factor family in cellular growth regulation. These data implicate Id family proteins as growth-promoting factors expressed in a wide variety of cells. The expression of their transcripts is associated with proliferative states and their downregulation is associated with differentiation and loss of proliferative potential (11,37,44,45,64). The wide distribution of Id family expression suggests a general and important role for Id proteins.

Id3 mRNA is undetectable in quiescent fibroblasts, but is sharply induced by mitogenic stimuli such as serum growth factors or protein kinase C stimulation by phorbol esters, with superinduction of Id3 mRNA in the presence of the protein synthesis inhibitor cycloheximide (7,8,10). Since Id class proteins appear to exert their effect primarily by interactions through the

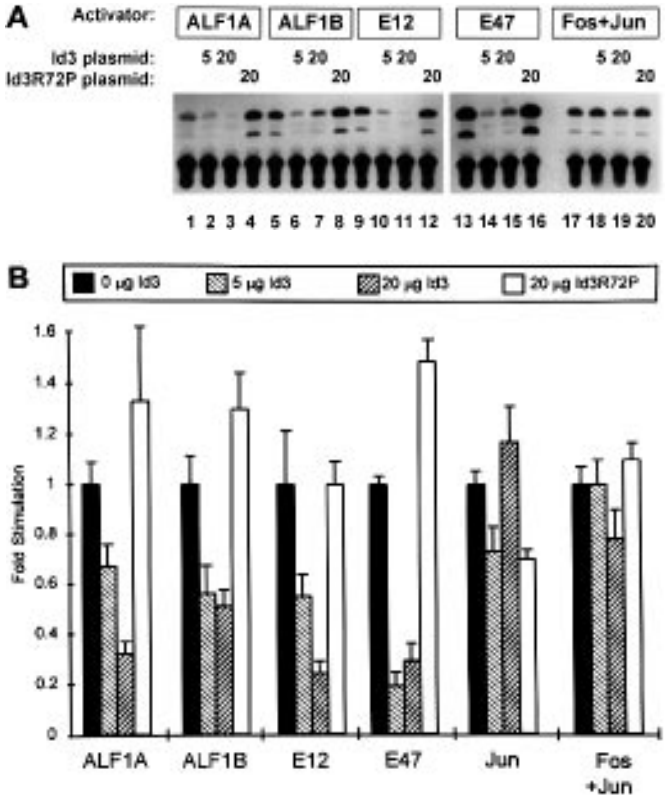

Figure 3. Id3 blocks reporter gene transactivation by bHLH proteins. (**A**) Autoradiogram of CAT assay. Plasmids expressing ALF1A, ALF1B, E12 or E47 were transiently transfected in varying amounts along with a reporter plasmid responsive to these bHLH proteins, $E(5+2)_4$CAT. As a control, similar amounts of Id3 or Id3R72P plasmid were cotransfected with c-Fos and c-Jun expression vectors and the c-Fos/c-Jun responsive reporter plasmid Coll-CAT. Cytoplasmic extracts were assayed for CAT activity on ^{14}C-chloramphenicol, and analyzed by thin layer chromatography and autoradiography. Data shown are representative of three to six transfection experiments for each data point. (**B**) Quantitation of CAT assays. Autoradiograms from multiple experiments from above were quantitated and normalized, with the activity of each activator without added Id3 defined as one. Dark bars indicate no added Id3, light shaded bars indicate 5 µg Id3 plasmid, dark shaded bars indicate 20 µg Id3 plasmid, and white bars indicate 20 µg mutant Id3R72P plasmid.

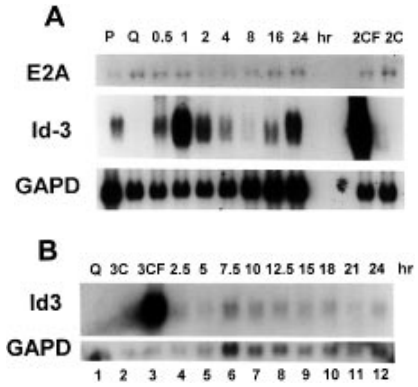

Figure 4. E2A mRNA expression is regulated during cell cycle re-entry. (**A**) Northern blot analysis of E2A and Id3 expression during cell cycle re-entry. Northern blots were prepared using 20 µg per lane of total RNA derived from BALB/c 3T3 fibroblasts. P, proliferating (lane 1); Q, quiescent (lane 2) or stimulated with 20% FBS for 0.5, 1, 2, 4, 8, 16 or 24 h (lanes 3–9) as indicated. Two additional samples were treated with cycloheximide 10 µg/ml for 2 h in the presence (2CF, lane 10) or absence (2C, lane 11) of 20% FBS. Blots were hybridized with radiolabeled E2A, Id3 or GAPD cDNA probes, and autoradiographed. (**B**) No additional regulation of Id3 mRNA is seen at S phase entry. A similar blot was prepared using 10 µg per lane of total RNA to evaluate Id3 expression focused at later time points. Q, quiescent (lane 1); 3C, treated with cycloheximide for 3 h (lane 2); 3CF, treated with cycloheximide and 20% FBS for 3 h (lane 3); or stimulated with 20% FBS for 2.5, 5, 7.5, 10, 12.5, 15, 18, 21 or 24 h (lanes 4–12).

HLH domain (65), it seems likely that the newly induced Id3 protein must interact with a protein that exists in serum-starved fibroblasts prior to mitogenic stimulus. Previously, the targets of Id proteins had been determined only in a candidate protein approach, testing bHLH proteins cloned for other reasons. Because of this, and because it seemed possible that the divergent determinants outside the HLH motif might contribute to differing activity or regulation, we undertook this library screen without preconceived notions. However, the limitations of this study are those of the two hybrid technique, and this may be characterized as an empiric, rather than exhaustive, investigation.

The results of this investigation primarily implicate the products of two E protein genes, E2A and ALF1, as physiologic targets of Id3. Interaction of Id3 with two alternative splice products from each of these genes is confirmed in the yeast two-hybrid assay, *in vitro* and in cultured mammalian cells. Our data show that E2A transcripts and proteins are upregulated in quiescent cells, further implicating E2A proteins as pre-existing, potential targets for growth factor induced Id3.

These results also show that Id3 binds to a third E protein, E2-2, and at least one additional bHLH protein, MyoD. These assays were performed with full length proteins where possible, in contrast with much of the published data investigating dimerization and DNA binding by these proteins. The use of full length proteins is potentially important due to published data showing that occasionally truncated bHLH proteins become more promiscuous with respect to their dimerization or DNA binding (60,61). In fact, the interaction of Id3 with Id4 detected in the two hybrid screen using truncated fusion proteins was not strongly supported in our coimmunoprecipitation studies using full length proteins *in vitro*. There has been no published evidence to suggest that interaction between Id proteins is important physiologically, although the potential for Id1 homodimerization has been described (66).

The molecular consequence of Id3 binding to E proteins is the formation of inactive heterodimers. Evidence of loss of E protein function is provided by DNA binding assays *in vitro* and transcriptional activation assays in transfected cells. This effect is dependent upon the integrity of the Id3 HLH motif. These findings are similar to data obtained for Id1 in many previous investigations (37,43,67). In fact, no published data has documented any clearly defined functional difference between any member of the Id protein family, despite their marked amino acid sequence divergence outside the HLH motif. It is not clear what, if any, function is served by the nonconserved regions outside the HLH. It has been proposed that the Id genes originally evolved by gene duplication, followed by mutational drift in regions of the proteins nonessential for Id function (68). According to this model, the sequence divergence outside the HLH indicates that these regions of the proteins serve no essential function.

Several lines of published evidence suggest that the balance between Id and bHLH proteins is critical to cell proliferation. First, expression of Id1 mRNA declines with the induction of

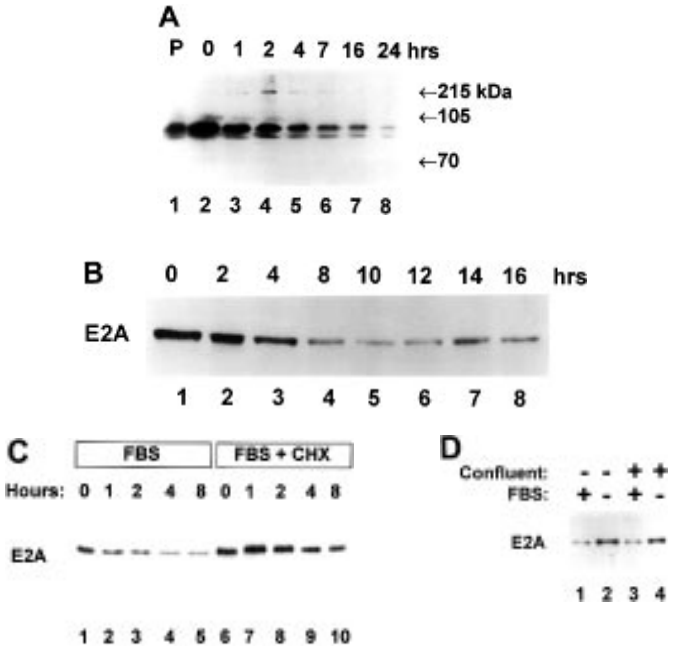

Figure 5. Regulation of E2A protein expression in fibroblasts. (**A**) E2A protein expression is downregulated during cell cycle re-entry. Immunoblots were prepared using nuclear extract from fibroblasts that were proliferating (P, lane 1), quiescent (0, lane 2), or stimulated with 20% FBS for 1, 2, 4, 7, 16 or 24 h (lanes 3–8) as indicated. The blot was probed with anti-E2A antibody. (**B**) No significant regulation of E2A occurs at S phase entry. Whole cell extracts were analyzed by immunoblot from fibroblasts that were serum starved (lane 1), or stimulated with serum for 2, 4, 8, 10, 12, 14 or 16 h as indicated (lanes 2–8). (**C**) E2A protein degradation is dependent upon new protein synthesis. Fibroblasts were serum starved for 72 h (lanes 1 and 6) and then stimulated with 20% FBS for 1, 2, 4 or 8 h in the absence (lanes 2–5) or presence (lanes 7–10) of cycloheximide. Whole cell extracts were analyzed by immunoblot for E2A content. (**D**) E2A expression is induced by serum starvation but not by contact inhibition of cell proliferation. Subconfluent (lanes 1 and 2) and confluent (lanes 3 and 4) fibroblasts were incubated in media containing 10% FBS (lanes 1 and 3) or 0.5% FBS (lanes 2 and 4). Whole cell extracts extracts were assayed by immunoblot for E2A content. Note that degree of exposure differed for each luminogram.

differentiation and loss of proliferation in myoblasts and hematopoietic cell lines (37,44,45). Second, enforced expression of Id1 mRNA results in loss of differentiation in these cell lines. The same is true of enforced Id1 expression targeted to B lymphocytes of transgenic mice (46). Third, enforced expression of Id2 causes accelerated S phase entry and cellular proliferation by a mechanism that appears to involve direct association with Rb, the product of the retinoblastoma locus (49). Fourth, antisense inhibition of Id1, Id2 and Id3 prevents S phase entry (47,48). Fifth, overexpression of E2A or MyoD proteins (Id target proteins) slows cell cycle progression or elicits frank growth arrest (63,69,70). Lastly, the data in this paper demonstrate that the E2A and Id3 genes are inversely regulated by soluble growth factors.

Regulated expression of E2A proteins

It is an important and novel finding in this paper that E2A expression is regulated significantly upon cell cycle re-entry of serum starved fibroblasts. The activities of other bHLH proteins such as MyoD previously have been implicated in cell cycle arrest (63,69,70). Peverali *et al.* have previously shown that induced overexpression of E2A proteins inhibits G_1–S cell cycle progression (63). However, ours is the first published evidence that E2A protein levels are naturally regulated according to proliferative status of the cell. This is consistent with the proposed regulatory role of E2A in cell proliferation (Fig. 4B). Thus far we have no data to indicate that Id3 or E2A directly regulate one another's promoters, although this remains a possibility.

Following serum stimulation, E2A steady state protein levels decline significantly. If E2A proteins had an intrinsically short half-life, then the rate of decline should be the same in cyclohemixide treated cells. However, our finding that the half life is dramatically prolonged in the absence of new protein synthesis indicates that E2A is a very stable protein, and that a newly synthesized protein factor must be responsible for its degradation following serum stimulation. We believe that this mechanism may involve the ubiquitin–proteasome pathway, as we have cloned a novel murine ubiquitin conjugating enzyme that binds to E2A proteins in the yeast two hybrid assay (D.A.L., T. S. Schaefer, M.B.S. and G.J.K., manuscript in preparation).

Our findings regarding E2A expression in untransfected fibroblasts demonstrate two important points. First, E2A mRNA and protein levels vary with proliferative status, suggesting that E2A plays a normal role in regulating cell growth. This shows that the results of Peverali *et al.* are not simply an artifact of induced overexpression of E2A proteins. Second, the downregulation of E2A proteins involves complex multifactorial mechanisms. The E2A steady state mRNA level is downregulated, either at the level of transcription or mRNA stability; E2A activity is antagonized by the induction of Id proteins; and very interestingly, E2A protein is degraded through a pathway requiring new protein synthesis. Accumulation of E2A proteins with growth factor withdrawal suggests that E2A might directly mediate cell cycle arrest due to absence of mitogen. Conversely, in tumorigenesis, loss of functional E2A genes might form part of a pathway for developing growth factor independence, and thus E2A might therefore constitute a tumor suppressor gene. In support of this idea, translocations of the E2A locus in human leukemia interrupt E2A function in at least one allele (71–74). In addition, E2A null mice that survive the newborn period have an extremely high propensity to develop lymphomas (C. Murre, personal communication). These findings suggest that the contribution of E2A chromosomal translocations in human cancer may be more complex than previously suspected.

In summary, all available data suggest that opposing molecular and biological roles are occupied by Id HLH proteins on one side, and by E proteins and other related bHLH proteins such as MyoD on the other. These bHLH proteins exert a growth suppressive effect upon the cell, presumably mediated at least in part by their transcriptional activation of growth suppressive genes. For example, MyoD has been shown to transactivate the gene encoding the p21 cyclin-dependent kinase inhibitor, causing growth suppression (75). It is conceivable that E2A exerts its growth suppressive effect by a similar mechanism, activating the p21 and potentially other growth suppressive genes. Although E2A homodimers are known to be able to bind and transactivate enhancers of the immunoglobulin heavy chain gene and the muscle creatine kinase gene, relevant growth regulatory targets remain to be identified.

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