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Identification of a novel transcriptional activity of mammalian Id proteins

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ABSTRACT

The Id proteins are a family of related mammalian helix–loop–helix (HLH) proteins which can interact with other HLH proteins but lack a basic region and are thus not thought to bind to DNA. Instead, they are hypothesized to act as dominant negative regulators of DNA-binding basic HLH (bHLH) proteins, by forming inactive heterodimers with these proteins. All four Id family proteins possess related HLH dimerization domains and can interact with similar bHLH proteins, although with differing affinities. The functions of the largely unrelated N- and C-terminal regions of the proteins are unknown. In this study, we have identified a novel transcriptional activity of the mammalian Id proteins. We show that when fused to the heterologous GAL4 DNA-binding domain, all four of the mammalian Id proteins can activate GAL4-dependent transcription. The HLH domain is necessary for the transactivation activity observed, suggesting that interaction with a cellular HLH protein is required. Co-transfection with exogenous Class A bHLH proteins (E-proteins) greatly potentiates the transactivation, which is abolished upon co-transfection with Class B bHLH proteins. These results are consistent with the idea that the Id proteins have a transcriptional activity when present in a DNA-binding complex.

INTRODUCTION

The helix–loop–helix (HLH) protein class of transcription factors are important regulators of cellular development and differentiation in a number of cell types (1). The HLH domain mediates interactions between members of this class of proteins, many of which function as heterodimers between a widely expressed (Class A) and a tissue-specific (Class B) basic HLH (bHLH) protein. The heterodimers bind to DNA through basic DNA-binding domains, usually to a related binding site called an E-box. A separate class of HLH proteins does not possess a basic region and is thus not thought to bind to DNA (2). The four mammalian members of this family (Id1–Id4) can, however, interact strongly with DNA-binding Class A bHLH proteins and more weakly with some of the Class B bHLH proteins (3). Since the Id proteins do not possess a basic region, dimers containing them are not thought to be able to bind DNA. Thus, they are hypothesized to act as dominant-negative regulators of interacting bHLH protein-mediated transcription. Although the four members of the mammalian Id protein family are related at the amino acid sequence level throughout the HLH domain (69–79% identity), the other parts of the proteins are not very related. It has been proposed that this group of proteins function mainly as negative regulators of bHLH protein-mediated transcriptional activity, by forming non-DNA-binding heterodimers with bHLH proteins and preventing their DNA binding and transcriptional activities (4). Many of the assays which are used to determine Id protein activity measure only binding to E-box sites or transactivation of E-box-dependent promoters, thus they cannot detect other activities that Id proteins might possess. We have investigated the possibility that the Id proteins can function in other ways besides merely titrating out the E-proteins in a cell. Here we report a novel activity of the mammalian Id family proteins; a transactivation activity observed when they are bound to DNA through fusion with the DNA-binding domain of the yeast transcriptional regulator GAL4. This activity requires an HLH region and thus probably depends on the ability of the Id proteins to interact with other HLH proteins in a cell. Addition of ectopic E-proteins increases transcriptional activation by the Id proteins, suggesting that E-proteins may be relevant dimerization partners mediating this phenomenon in cells. These results support a hypothesis that Id proteins may play roles other than just as dominant negative regulators of bHLH protein function.

MATERIALS AND METHODS

Plasmid construction

Mammalian GAL4 fusions. GAL4–Id1 was constructed by inserting a 900 bp *Hin*dIII fragment containing full-length mouse Id1 cDNA into the *Hin*dIII site of pM3 (5). GAL4–Id1 13–88 contains only amino acids 13–88 of Id1 and was constructed by subcloning a *Pst*I fragment into pM2 (5). GAL4CTId1 contains the C-terminal amino acids 99–148 from Id1 inserted into pM3, while GAL4ΔCTId1 contains amino acids 1–99 inserted into pM3. GAL4–Id2 was constructed by fusing the full-length coding

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region of mouse Id2 cDNA to the GAL4 DNA-binding domain of pM3. For construction of GAL4–Id3, the full-length coding sequences of mouse Id3 was produced by PCR and inserted into the *Bam*HI site of the pSG424 vector (6). GAL4–Id3 deletion constructs were produced by inserting the appropriate truncated fragments generated by PCR into pSG424. GAL4ΔNId3 lacks the N-terminus of Id3 and has amino acids 2–40 deleted, GAL4ΔCId3 lacks the C-terminus of Id3 (amino acids 82–119 are deleted), while GAL4ΔHLHId3 lacks the HLH region of Id3 (amino acids 41–81). GAL4HLHId3 contains only the HLH region of Id3 (amino acids 41–81), while GAL4CTId3 contains only the C-terminus of Id3 (amino acids 82–119). GAL4–Id4 contains the full-length coding region of Id4 in pM3. GAL4E1A was described previously (7).

Other plasmids. CMVMyoD (8) and the reporter constructs G5E1bCAT (7), G5E1bLUC (9), G5TKCAT and BL2CAT (10) were described previously. For construction of CMVdnME1, the bHLH region and C-terminus of ME1 was generated by PCR and inserted into pCEP4F(–EBNA) (11).

Cell culture

293 human embryonal kidney cells were maintained in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml of penicillin and streptomycin. CV1 African green monkey kidney cells were grown in OPTI-MEM (Gibco BRL) supplemented with 5% FBS and 100 μg/ml of penicillin and streptomycin.

Transient transfection assays

All transfections were done using a calcium phosphate precipitation kit (5′-3′, Boulder, CO). 293 cells were plated at 2×10^5 cells/35 mm plate ~24 h prior to transfection. CV1 cells were plated at a density of 2×10^5 in 60 mm plates. Each plate was co-transfected with luciferase or CAT reporter plasmid, cDNA expression construct(s) and RSV-βgal (12). Cells were harvested 36 h after transfection and assayed for luciferase (Luciferase Assay System, Promega Corp.) or CAT activities (13). β-Galactosidase activity was measured using a kit (Galacto-Light, Tropix Inc.); luciferase or CAT activities were normalized to the β-galactosidase activity in the same sample to control for differences in transfection efficiency. Each transfection was repeated a minimum of four times.

Western blot analysis

293 cells were transfected and harvested as described above. After analysis of luciferase or CAT reporter activity, the cell lysates and pellet were combined and lysed further in 1% SDS. Protein concentrations were determined and ~50 μg of each was electrophoresed. In some experiments, cells were treated with 5 μM Z-L_3VS proteasome inhibitor 24 h after transfection and harvested 24 h later. Cells were harvested and lysed in 100 μl of 100 mM Tris, pH 7.5, and 100 μl of 1% SDS. For all western blots, ~50 μg of protein were loaded onto 10–12% SDS gels, transferred to nitrocellulose and detected with 0.2 μg/ml anti-GAL4 DNA-binding domain antibody (Santa Cruz Biochemical, Santa Cruz, CA). Detection of antigen–antibody complexes was performed using alkaline phosphatase (Kirkegaard & Perry Laboratories, Gaithersburg, MD) according to the manufacturer's protocol.

RESULTS AND DISCUSSION

Id proteins have transactivation activity

Id family proteins are thought to act as negative regulators of bHLH proteins and their transcriptional activities. It has been assumed that the non-HLH portions of Id proteins are largely dispensable for their activity, although a few reports have suggested that the non-HLH regions may contribute to Id protein function (14,15). Interestingly, we have found that when the full-length coding regions of the Id proteins are fused to the heterologous GAL4 DNA-binding domain, they activate transcription of a GAL4-responsive reporter gene. Human 293 cells were co-transfected with the reporter plasmids G5TKCAT (TK promoter with five GAL4 binding sites upstream, GAL4-responsive) or BL2CAT (TK promoter without the GAL4 sites, non-GAL4-responsive), along with the GAL4–Id protein fusions (GAL4–Id1, GAL4–Id2, GAL4–Id3 or GAL4–Id4). Co-expression of GAL4–Id fusion proteins with the reporter plasmid lacking GAL4 binding sites caused very little transcriptional activity (Fig. 1). However, co-expression of the GAL4–Id fusion proteins with the GAL4-responsive reporter plasmid (G5TKCAT) significantly increased activation of the reporter gene (Fig. 1). The transactivation activity of the GAL4–Id proteins was comparable with that of the GAL4–E1A fusion protein (Fig. 1), which contains a strong activation domain derived from the adenovirus E1A protein (7). The GAL4–E1A fusion used contains amino acid residues 121–223 of E1A, which contains conserved regions 2 and 3 and posesses strong transactivation activity (7). We also observed similar transactivation activities with a GAL4–E2A fusion which contains the E2A coding region fused to the GAL4 DNA-binding domain (data not shown). Although the GAL4–Id1 fusion protein appears to activate GAL4-dependent transactivation more strongly than the other GAL4 fusion proteins; this may reflect the higher level of expression of this construct following transfection. Western blot analysis of cellular extracts prepared from transfected cell cultures indicated that GAL4–Id1 is expressed at higher levels than GAL4–Id2, GAL4–Id3 and GAL4–Id4 (Fig. 1B). GAL4–Id1 is expressed at the highest level, followed by GAL4–Id4; GAL4–Id2 is expressed, but at a low level. We were unable to detect the expression of either GAL4–EIA or GAL4–Id3 in this experiment, although they possess strong transactivation activity. Our attempts to immunoprecipitate the GAL4 fusion proteins using this antibody were not successful, thus we treated the cells with a proteasome inhibitor to attempt to increase the amount of fusion protein present. 293 cells were transiently transfected and treated before harvest with Z-L_3VS, a covalent, irreversible inhibitor of the 26S proteasome (16) before western blot analysis. As shown in Figure 1C, under these conditions GAL4–Id1, GAL4–Id2 and GAL4–Id4 proteins were readily detectable, but GAL4–E1A and GAL4–Id3 proteins were not, despite their strong transactivation activity. We conclude that they are present at low levels only detected by the sensitive reporter assays.

The GAL4 DNA-binding domain fusion assay has been used extensively to define the transcriptional activation domains of numerous proteins (17,18). There are many examples of transcriptionally active proteins which need to be tethered to DNA through their fusion to or interaction with a protein containing a DNA-binding domain. A well-known example of this is the VP16 protein of herpes simplex virus (reviewed in 19). Although VP16 does not strongly bind to DNA on its own, it possesses a very

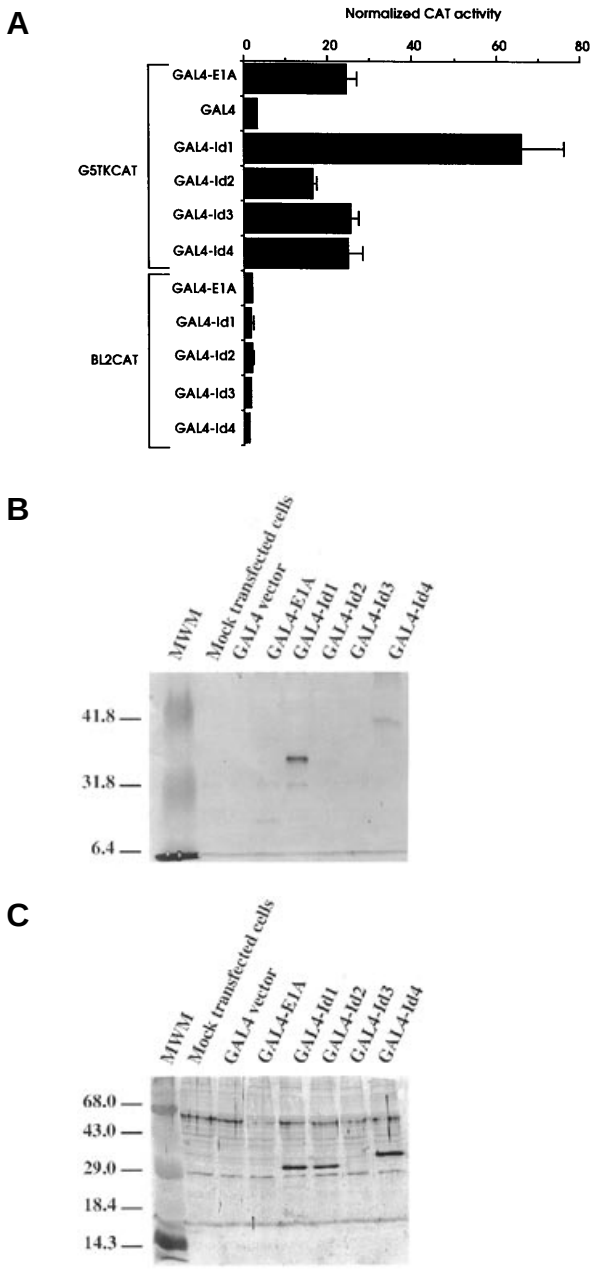

Figure 1. Id proteins have transactivation activity. (**A**) 293 cells were co-transfected with 2 µg of G5TKCAT or BL2CAT and 1 µg pRSV-βgal reporter plasmids, together with 1.5 µg of the indicated GAL4 DNA-binding domain plasmids (GAL4–Id1, GAL4–Id2, GAL4–Id3, GAL4–Id4 or GAL4–E1A). An aliquot of 1.5 µg of pBluescript KS DNA was added to bring the amount of DNA in each transfection to 5 µg. GAL4, GAL4 DNA-binding domain (DBD) alone, not fused to anything. To correct for differences in transfection efficiency, CAT activities were normalized to β-gal activities in the same extract. CAT activities are presented as the means of duplicate samples ± the standard deviation. Results of a representative experiment are shown; each transfection was repeated a minimum of four times. (**B**) Western blot analysis of cell extracts from 293 cells transfected as described in (A). The cell lysates were prepared as described in Materials and Methods and ~50 µg of protein was loaded in each lane and electrophoresed on a 10% SDS–PAGE gel, then transferred to nitrocellulose. GAL4 fusion proteins were detected using anti-GAL4 DBD antibody (0.2 µg/ml). (**C**) 293 cells were transfected as described in (A) and treated with 5 µM of the proteasome inhibitor, Z-L_3VS, for 24 h before harvest. Approximately 50 µg of protein was loaded in each lane and electrophoresed on a 12% SDS–PAGE gel, transferred to nitrocellulose and incubated with anti-GAL4 DBD antibody as described above.

strong activation activity when fused to the DNA-binding domain of GAL4. In cells, VP16 interacts with the widely expressed Pou homeodomain protein Oct1 and to a host cell factor; this interaction changes the specificity of the Oct1 DNA binding (19). The finding that the Id proteins can activate transcription when tethered to DNA via the GAL4 DNA-binding domain was surprising, since this class of proteins was originally postulated to function solely by interacting with and repressing DNA-binding of bHLH proteins, although some evidence suggests that they possess other activities (15,20,21). The observation that Id proteins possess a transcriptional activation capability of their own implies that they may perform more complicated functions than previously suggested.

HLH domains of Id1 and Id3 are necessary for transactivation function

To determine which domains of Id1 and Id3 are necessary for the observed activation activity, we fused different portions of Id1 or Id3 to the GAL4 DNA-binding domain and tested the ability of these fusion proteins to activate transcription of the GAL4-dependent reporter gene G5E1bLUC or G5TKCAT. As shown in Figure 2A, the HLH region and the C-terminus of Id3 are required for its transactivation activity, as deletion of either of these domains abolishes transactivation. Deletion of the N-terminus of Id3 did not affect its ability to activate transcription, suggesting that the N-terminus is not necessary for this activity. In contrast to Id3, a GAL4–Id1 fusion protein lacking the Id1 C-terminus was still able to activate transcription, although to a reduced extent relative to full-length Id1 protein (Fig. 2B). A GAL4–Id1 fusion protein in which the N-terminus, HLH domain and C-terminus were all disrupted (GAL4–Id1 13–88) did not activate transcription to an appreciable extent (Fig. 2B). The Id1 C-terminus alone produced no significant activation of the reporter gene. To confirm that the above results reflected the differences in the transactivation activity of the various GAL4–Id1 mutants and not lack of protein expression, we analyzed the cellular extracts of cells transfected with the GAL4–Id1 mutants by direct western blotting. We were unable to perform similar analysis with GAL4–Id3 mutants since we could not detect GAL4–Id3 using the anti-GAL4 antibody. Figure 2C shows that all GAL4–Id1 derivatives are detectably expressed; GAL4–Id1 13–88 appeared to be expressed at the lowest level. Taken together, these results suggest that the HLH regions of the Id proteins are important for their ability to activate transcription. Other regions of the proteins also appear to contribute, at least in the case of Id3, where the C-terminus is required as well. Interestingly, the C-terminus of Id3 is also required for the ability of Id3 to inhibit muscle cell differentiation (14). We have recently found that the C-terminus of Id3 interacts with a putative coactivator protein (M.A.Bounpheng *et al.*, manuscript submitted), which may contribute to its activation potential. However, the current results show that an intact HLH region is also necessary for transactivation activity by Id3. This suggested to us that the Id proteins may be activating transcription through interactions with another HLH protein containing a transactivation domain, by bringing it into proximity with the GAL4-responsive promoter.

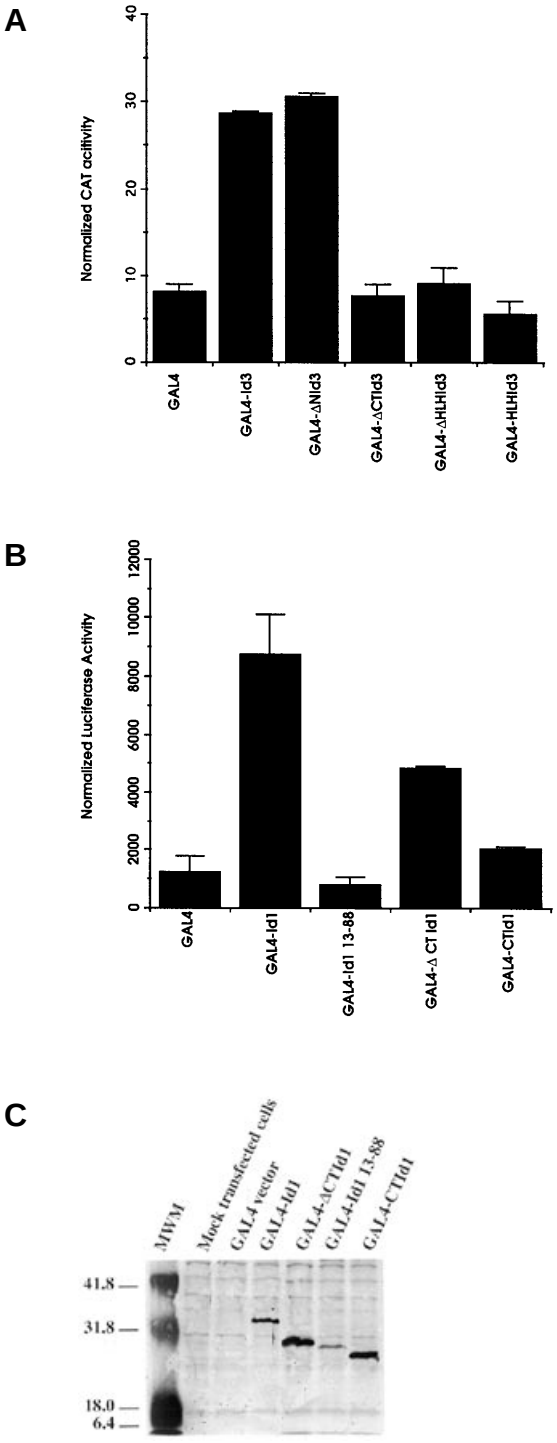

Figure 2. The HLH domain is necessary for the transactivation activity of GAL4–Id1 and GAL4–Id3 fusion proteins. (**A**) CV1 cells were co-transfected with 2 µg of G5TKCAT and 1 µg of pRSV-βgal reporter plasmids, along with 2 µg of the indicated GAL4–Id3 fusion protein expression constructs. GAL4, GAL4 DNA binding domain alone; GAL4–Id3, full-length Id3 coding region fused to the GAL4 DBD. GAL4ΔNId3, GAL4ΔCTId3 and GAL4ΔHLHId3 are fusions between the GAL4 DBD and the truncated Id3 coding region as described in the text. To correct for differences in transfection efficiency, CAT activities were normalized to β-gal activities in the same extract. CAT activities are presented as the means of duplicate samples ± the standard deviation. The results of a representative experiment are shown; each transfection was repeated a minimum of four times. (**B**) 293 cells were co-transfected with 1 µg G5E1bLUC and pRSV-βgal reporter plasmids, together with 2 µg of the indicated GAL4–Id1 fusion protein expression constructs. GAL4–Id1, full-length Id1 coding region fused to GAL4 DBD; GAL4–Id1 13–88, GAL4–ΔCTId1 and GAL4–CTId1 are fusions between the GAL4 DBD and the truncated Id1 coding region as described in text. Luciferase activities were normalized to β-gal activities in the same extract to control for differences in transfection efficiency. Results of a representative experiment are shown; each transfection was repeated a minimum of four times. (**C**) Western blot analysis of cell extracts from 293 cells transfected with GAL4–Id1 derivatives. Approximately 50 µg of each protein sample was fractionated on a 10% SDS–PAGE gel and transferred to nitrocellulose. GAL4 fusion proteins were detected with the anti-GAL4 DBD antibody at a concentration of 0.2 µg/ml.

Augmentation of GAL4–Id protein transactivation activity by E-proteins

If the observed transactivation activity of Id proteins is at least due in part to their ability to interact with other HLH transcription factors that supply a transactivation domain to the complex, then it should be possible to increase the transactivation activity of Id proteins by adding an exogenous Id-interacting transactivator protein to the cells. It is possible that the activity that we observed with GAL4–Ids transfected alone was due to their ability to complex with the endogenous activator proteins. In transfected cells, such proteins would be present in a limiting amount relative to the exogenous GAL4–Id protein and thus the observed activation activity would be low. It has been demonstrated previously that the mammalian Id proteins can interact with the widely expressed bHLH proteins belonging to the E2A protein family (E-proteins) (3). These proteins, possessing transcriptional activities of their own, can bind DNA as homodimers or as heterodimers with tissue-restricted bHLH factors (1). Initially, we tested the ability of the GAL4–Id fusion proteins to interact with a member of the E-protein class, ME1, using the mammalian two-hybrid assay. ME1 (also known as Alf-1 and HEB/REB) is a bHLH protein which is highly expressed in the nervous system (18,22–25). The ME1 gene gives rise to two alternatively spliced variants, ME1a and ME1b. ME1a and ME1b, which have some differences in transcriptional activity, differ by the presence of a 24 amino acid ankyrin repeat domain (23). However, both versions have been found to activate as well as repress transcription, depending on the promoter context (26). All GAL4–Id fusion proteins tested were able to interact with a partial ME1 cDNA fused to the VP16 activation domain in the mammalian two-hybrid assay (data not shown). We tested the ability of ME1a and ME1b to affect transcriptional activation of the GAL4–Id1 or GAL4–Id3 fusion proteins. In this assay, the ME1 proteins are not fused to the VP16 transactivation domain. As shown in Figure 3A, co-transfection of either ME1a or ME1b along with GAL4–Id1 increased the GAL4-dependent transcriptional activation ~8- to 10-fold. No significant differences in activity in this assay were detected between ME1a or ME1b. Two other E-proteins (E12 and E47) were also tested in this assay and gave similar results to those obtained with ME1 (data not shown). Both E12 and E47 were able to increase GAL4–Id1 activation by 20–60-fold; E12 appeared to be the strongest activator of GAL4–Id1 transcription (data not shown). These results are consistent with a model in which the Id family proteins activate transcription when bound to DNA by virtue of complexing with another transcription factor which contains a transcriptional

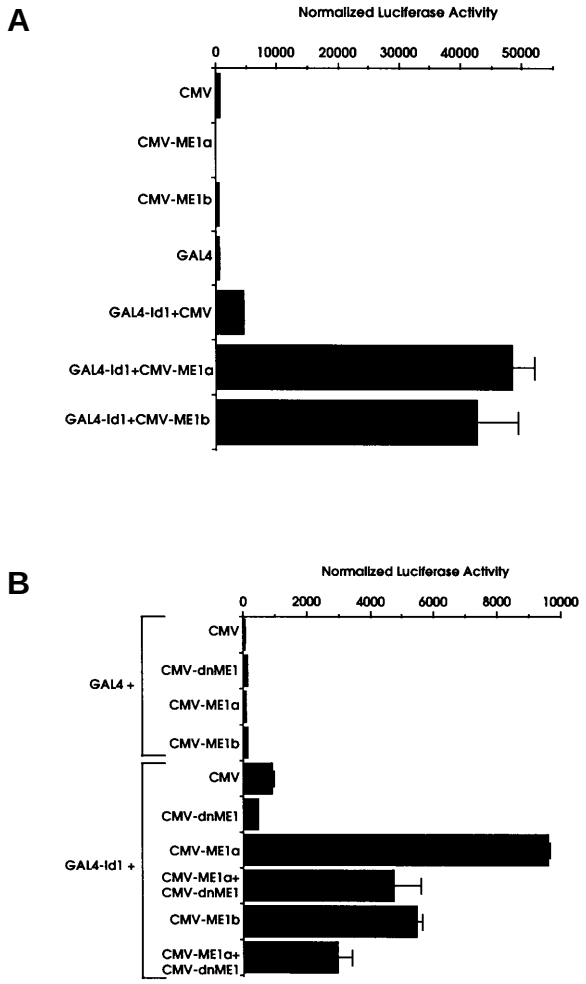

Figure 3. Addition of E-proteins increases activation by GAL4–Id1 fusion protein. 293 cells were transiently co-transfected with 2 µg G5E1bLUC, along with 1.5 µg each of the indicated expression constructs and 1 µg of pRSV-βgal. (**A**) GAL4–Id1 fusion protein was co-transfected with full-length ME1a or ME1b; addition of any of the Class A E-proteins increased the amount of GAL 4-dependent activation. ME1a or ME1b alone did not activate the GAL4-responsive promoter. (**B**) Addition of a non-activating mutant ME1 protein does not increase activation by GAL4–Id1 and can inhibit augmentation by the wild-type ME1 protein. Luciferase activities were normalized to the β-gal activity in the same sample to correct for differences in transfection efficiencies. At least four independent experiments were carried out for each sample; results of a representative experiment are shown.

activation domain and brings that activation domain into the proximity of the GAL4-dependent promoter. This suggests the possibility that although the Id proteins do not possess a recognizable DNA-binding domain, they might be present in DNA-binding protein complexes *in vivo*, thereby affecting transcription.

If GAL4–Id proteins are activating transcription by interacting with and bringing to the GAL4-responsive promoter a factor which possesses a transcriptional activation domain, then deletion of the activation domain of that protein should abolish its ability to augment GAL4–Id activation activity. We tested this by constructing an ME1 protein which lacked the N-terminus. When fused to the GAL4 DNA-binding domain, this part of ME1 was unable to activate GAL4-dependent transcription (data not shown). Since this construct contains the bHLH region, it should be able to interact with Id proteins and therefore was predicted to act as a dominant negative regulator of GAL4–Id activity. Following co-transfection of the truncated ME1 along with GAL4–Id1 into the cells, a decrease in GAL4-dependent transactivation is seen (Fig. 3B). When both the full-length and the truncated ME1 constructs were co-transfected together with GAL4–Id1 and the GAL4-responsive reporter gene, the truncated ME1 suppressed the augmented activation of the full-length ME1 (Fig. 3B). Thus, these results are consistent with our hypothesis that the Id proteins can activate transcription when tethered to DNA by virtue of interaction with transcriptional activator proteins. Addition of a transcriptionally inactive ME1 inhibits the ability of GAL4–Id1 to activate transcription, presumably by competing with available E-proteins (transfected or endogenous) for binding to GAL4–Id1. Similar results were obtained when truncated ME1 was co-transfected along with the GAL4–Id2, GAL4–Id3 and GAL4–Id4 proteins (data not shown).

The HLH domain of Id1 or Id3 is necessary for the augmented activation by E-proteins

Using deletion mutants of GAL4–Id1 and GAL4–Id3, we tested whether the HLH region was required for the increase in transactivation seen upon co-transfection of ME1. As shown in Figure 4A, co-transfection of ME1 augmented the transcriptional activation mediated by GAL4–Id1 (full-length Id1) and GAL4ΔCTId1 (C-terminus deleted), both of which contain intact HLH domains. Co-transfection of ME1 did not augment activation mediated by GAL4–Id1 13–88 or GAL4CTId1, neither of which contain intact HLH domains. Similarly, ME1a was able to augment transactivation only of those GAL4–Id3 fusions which contain an intact HLH domain. As shown in Figure 4B, co-transfection of ME1a increased transactivation by GAL4–Id3 (full-length Id3) and GAL4ΔNId3 (N-terminus deleted), both of which contain intact HLH domains. Co-transfection of ME1a did not augment transactivation by GAL4ΔHLHId3 (HLH deleted) or GAL4CTId3 (C-terminus alone), neither of which contains HLH domains. In the case of Id3, the C-terminus of the protein also appears to be required, since co-transfection of ME1a did not stimulate transactivation by GAL4ΔCTId3, which has the C-terminus of the protein deleted. These data, along with the data shown in Figure 2B, support our hypothesis that the Id proteins are able to activate transcription by interacting with a bHLH protein, most likely a member of the E-protein family. This implies that when Id proteins are complexed with proteins which contain DNA-binding domains, they may not act solely in a dominant negative fashion.

Alternative explanations for the ability of the Id family proteins to activate transcription and the ability of the E-proteins to augment this activation also exist. One possibility is that the Id proteins can activate transcription by virtue of interacting with a coactivator protein or by interacting directly with the basal transcriptional machinery. Thus far, no direct interactions between Id proteins and the basal transcriptional machinery have been demonstrated. However, we have recently isolated a c-Jun coactivator protein using the yeast two-hybrid screen with the Id3 protein as bait (M.A.Bounpheng *et al.*, submitted for publication). This coactivator protein can interact specifically with the Id3 and

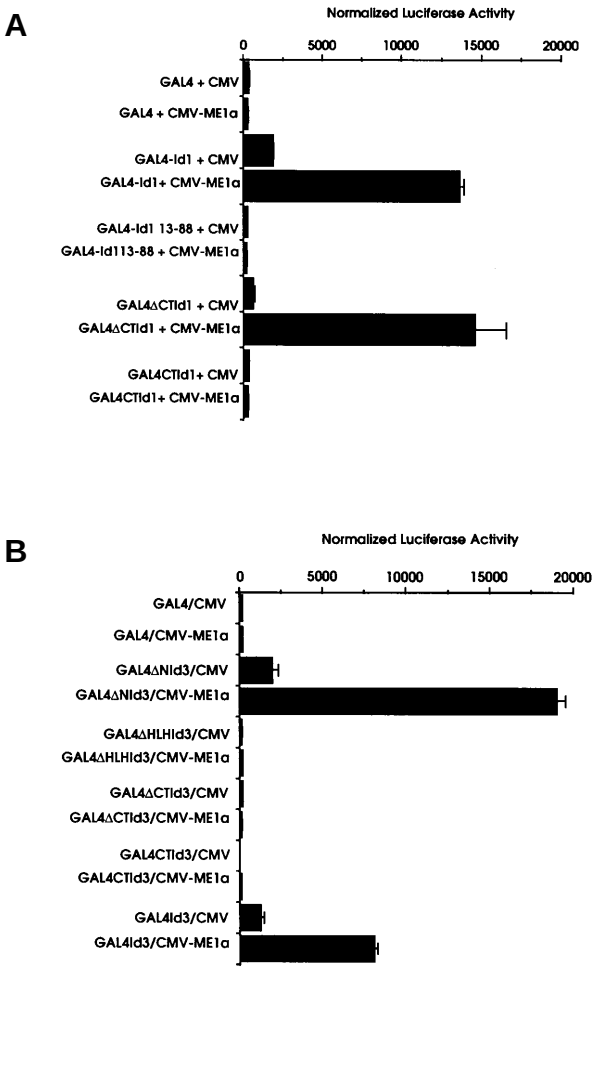

Figure 4. The HLH domain of the Id3 and Id1 proteins is necessary for augmentation of transactivation by E-proteins. 293 cells were transiently co-transfected with 2 µg of G5E1bLUC and 1 µg of pRSV-βgal reporter genes, along with 1.5 µg each of ME1 and the indicated full-length or truncated GAL4–Id1 or GAL4–Id3 constructs (described in text). At least four independent experiments were carried out for each sample; results of a representative experiment are shown. (**A**) GAL4–Id3 fusions. (**B**) GAL4–Id1 fusions. GAL4, GAL4 DNA-binding domain (DBD) alone; CMV, CMV expression vector alone.

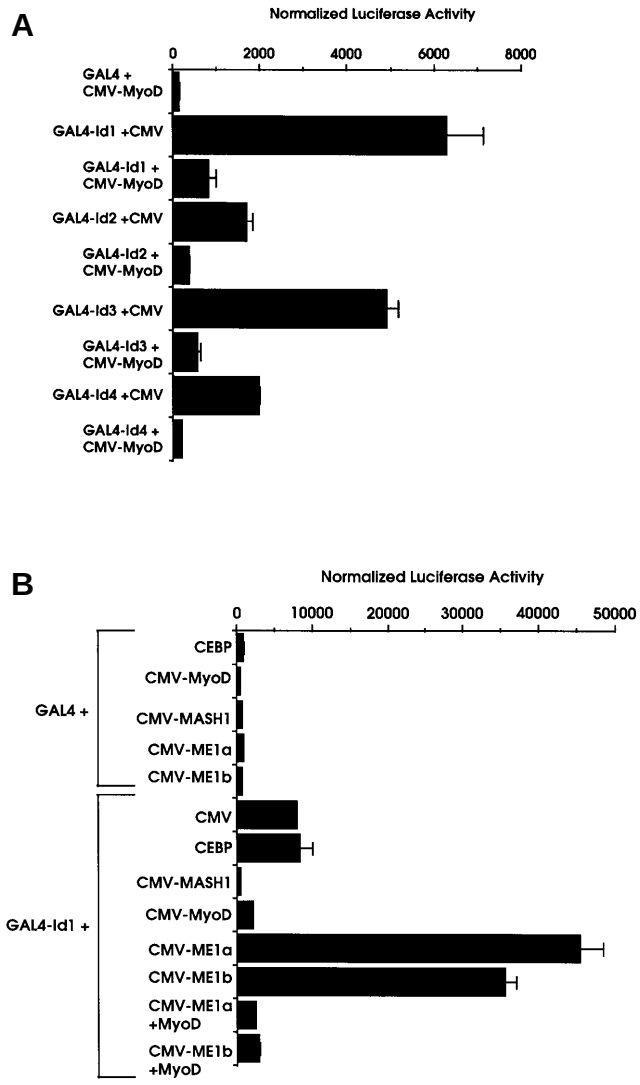

Figure 5. The Class B bHLH protein MyoD inhibits the transactivation activity of GAL4–Id fusion proteins and the augmentation of activation activity by E-proteins. 293 cells were transiently co-transfected with 2 µg of G5E1bLUC and 1 µg of pRSV-βgal reporter plasmids, along with 1.5 µg each of the indicated cDNA expression plasmids and GAL4–Id fusion protein expression plasmids. At least four independent experiments were carried out for each sample; results of a representative experiment are shown. (**A**) Co-transfection with MyoD abolishes GAL4-dependent transactivation by any of the GAL4–Id fusion proteins. (**B**) Addition of MyoD inhibits the augmentation of GAL4–Id1 fusion transactivation by the E-protein ME1. Addition of a different Class B bHLH protein, Mash-1, also abolishes GAL4-dependent transactivation by GAL4–Id1, but the leucine zipper transcription factor CEBP (which does not interact with the Id proteins) does not.

Id1 proteins in mammalian cells, but does not interact with Id2 or Id4 protein. It remains to be seen whether interactions with coactivators can explain the current observations. An alternative explanation for the ability of co-transfected E-proteins to increase Id protein activation activity is that they can increase the stability of GAL4–Id proteins. Further experiments are in progress in our laboratory to clarify this issue.

Addition of the Class B bHLH protein MyoD abolishes activation by Id proteins

If the Id proteins need to interact with the widely expressed bHLH factors to activate transcription, then addition of a bHLH protein which can compete with Id proteins for binding to an E-protein should inhibit the augmented activation which is presumably mediated by an interaction between Id proteins and the endogenous E-proteins. Addition of a large amount of such a bHLH protein should compete with Id protein for interacting with endogenous E-protein. In Figure 5A, we show that co-transfection of GAL4–Id fusion proteins with the bHLH protein MyoD abolishes the ability of all four GAL4–Id proteins to activate transcription. These data support our notion that the ability of the GAL4–Id

proteins to transactivate might require interactions with E-proteins. Although Id proteins interact weakly with MyoD family proteins, MyoD and the related proteins strongly interact with the E-proteins (27–29). MyoD may thus inhibit the activity of GAL4–Id proteins by sequestering endogenous E-proteins. As predicted, MyoD can also inhibit both the CMV–ME1a and CMV–ME1b augmentation of GAL4–Id1 activity (Fig. 5B). Using a similar assay, we found that MyoD can also inhibit the potentiation of GAL4–Id1 transactivation by other E-proteins, E12 and E47 (data not shown). If our hypothesis is correct, then co-transfection of other Class B bHLH proteins which interact with the E-proteins should also abolish the transactivation by the GAL4–Id fusion proteins and the potentiation of that activation by E-proteins. We have tested two other Class B bHLH proteins, eHAND and MASH-1. The results are similar to those reported here with MyoD (data not shown).

In summary, we have identified a novel transcriptional activation activity present in the mammalian Id proteins. When bound to DNA, all four Id proteins can activate transcription. This raises the possibility that the function of these proteins is more complicated than previously proposed. Inside the cell, these proteins may participate in DNA-binding complexes by virtue of interactions with other (DNA-binding) proteins, where they may contribute to transcriptional activity of the complex formed. Since the HLH domain of the Id1 and Id3 proteins are necessary for the observed transcriptional activation, we hypothesize that the activation may result from interaction with a bHLH protein inside the cell. Addition of a known interacting bHLH protein such as ME1, E12 or E47 increases the activation by DNA-bound Id proteins, suggesting that these Id-interacting factors may play a role in this process inside the cell. Consistent with this idea, co-transfection of GAL4–Id fusion proteins with Class B bHLH proteins (which interact strongly with the E-proteins, but only weakly or not at all with the Id proteins) abolishes Id transactivation activity. In this case, it is possible that Class B bHLH factors titrate out the available E-proteins inside the cell, rendering them no longer able to bind to the Id proteins. Although interactions of Id family proteins with bHLH proteins appear to inhibit binding of the latter to some E-box sites, it remains to be seen whether Id proteins can exist in complexes which work through other motifs.

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