Differential Interactions of Id Proteins with Basic-Helix-Loop-Helix Transcription Factors*

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Dimerization of three Id proteins (Id1, Id2, and Id3) with the four class A E proteins (E12, E47, E2-2, and HEB) and two groups of class B proteins, the myogenic regulatory factors (MRFs: MyoD, myogenin, Myf-5 and MRF4/Myf-6), and the hematopoietic factors (Scl/Tal-1, Tal-2, and Lyl-1) were tested in a quantitative yeast 2-hybrid assay. All three Ids bound with high affinity to E proteins, but a much broader range of interactions was observed between Ids and the class B factors. Id1 and Id2 interacted strongly with MyoD and Myf-5 and weakly with myogenin and MRF4/Myf-6, whereas Id3 interacted weakly with all four MRFs. Similar specificities were observed in co-immunoprecipitation and mammalian 2-hybrid analyses. No interactions were found between the Ids and any of the hematopoietic factors. Each Id was able to disrupt the ability of E protein-MyoD complexes to transactivate from a muscle creatine kinase reporter construct in vivo. Finally, mutagenesis experiments showed that the differences between Id1 and Id3 binding map to three amino acids in the first helix and to a small cluster of upstream residues. The Id proteins thus display a signature range of interactions with all of their potential dimerization partners and may play a role in myogenesis which is distinct from that in hematopoiesis.

An increasingly important role is ascribed to protein-protein interactions in the regulation of cellular growth and differentiation pathways. Dimerization serves to convert inactive monomeric molecules into transcriptionally active dimeric complexes at specific times during cellular development. Deletional analysis has identified a number of evolutionarily conserved regions that mediate these interactions. One such region, commonly associated with transcription factors involved in a range of proliferative and differentiation pathways, is the basic-helix-loop-helix (bHLH)1 (1). This domain is conserved from yeast to mammals and is composed of a positively charged basic region followed by two amphipathic alpha-helices separated by a spacer loop. Dimers are stabilized by a series of hydrophobic and electrostatic interactions between the helices of compatible molecules (2–5). The juxtaposition of two basic regions resulting from dimerization forms a DNA binding interface able to insert into the major groove in a sequence-specific manner (2, 6). Although bHLH proteins have no discernible DNA binding activity as monomers, dimers recognize a consensus DNA sequence (CANNTG), termed the E box (4, 7–9).

bHLH transcription factors can be broadly placed into two categories (reviewed in Ref. 10). The class A factors, or E proteins, (E2-2, HEB, and the E2A gene products E12 and E47) are expressed in a virtually ubiquitous pattern and are able to dimerize efficiently with tissue-restricted class B factors to activate gene expression (1, 11–13). Because each factor contributes a specific DNA recognition half-site, class A and class B heterodimeric complexes and class A hetero- or homodimers theoretically provide distinct combinatorial E box binding specificities (7). Class B members thus far characterized include the myogenic regulatory factors (MRFs) involved in skeletal muscle development (MyoD, myogenin, Myf-5, and MRF4/Myf-6) and the hematopoietic factors (Scl-Tal-1, Tal-2, and Lyl-1) (14–21). Mammalian homologues of the Drosophila bHLH achaete scute genes (ash1 and -2) have been implicated in neuronal development as has neurod (beta2) which also has a role in insulin regulation (22–24). A range of other class B bHLH proteins have been associated with early mesoderm formation and later muscle development (Twist), adipocyte development (Add1), and skeleton formation (Scl-1) (25–27). E box motifs have been identified in the enhancer elements of a number of bHLH factor-regulated genes such as myosin heavy chain, Immunoglobulins, and chymotrypsin (10).

The formation of active class A-class B complexes is modulated by the Id (inhibitor of DNA binding) family members. The four Id proteins identified thus far have an HLH domain that lacks the amino-terminal associated basic region necessary for DNA binding (28–33). Id proteins act to sequester class A factors, inhibiting the formation of active class A-class B heterodimers and are therefore considered to act as dominant negative regulators of differentiation pathways (28, 34, 35). Ids are expressed in a largely overlapping but distinct fashion during development, with the highest levels generally being achieved during embryogenesis (33). Significant Id levels persist in a range of actively proliferating tissues and in some tumor cell lines (28, 33). Rapid Id down-regulation has been reported in myoblasts and hematopoietic cells during terminal differentiation, consistent with their negative regulatory role (28, 34). Indeed, forced Id expression has been shown to inhibit the differentiation of each of these cell types (36–40). Because considerable overlap exists in the expression patterns of Id proteins, redundancy in their function has been inferred (30, 33).

Ids are known to bind avidly to class A factors such as E47, weakly to the myogenic factors, and poorly, if at all, to the hematopoietic factors (28, 34, 41). This has led to the assump-
tion that transcriptional control is exerted primarily at the level of Id-class A interactions. However, these studies were performed with select members of the class A and class B families. Thus far, no exhaustive studies exist to compare the relative strengths of interactions among a broader range of family members.

The yeast 2-hybrid system has become an increasingly popular method for assessing protein-protein interactions and has been employed previously to study a subset of bHLH interactions (35, 42–44). We sought to develop a quantitative yeast 2-hybrid assay to investigate interactions of three Id proteins with a range of class A and class B factor targets. Our observations, confirmed and extended by co-immunoprecipitation (IP) of in vitro translated proteins and mammalian 2-hybrid analyses, indicate that discrete and reproducible differences exist in relative binding preferences among the Id proteins. As expected, Id proteins bound avidly to all the class A factors tested, although a range of affinities were apparent. A broader range of affinities for myogenic factors was observed, and no interactions with the hematopoietic factors were seen despite their expression in functional form.

Transient transfection studies in C3H myoblasts employing a muscle-specific creatine kinase chloramphenicol acetyltransferase (CAT) reporter vector provided independent confirmation of the hierarchical interactions of Id proteins with class A factors. Site-directed mutagenesis enabled us to map the regions responsible for establishing Id dimerization preferences to the first helix of the HLH domain and to residues immediately adjacent to this. Our findings have implications for the mechanism by which Id proteins influence class A and class B interactions and for the roles played by different Id proteins in tissue-specific gene regulation.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Parental yeast vectors pGBT9 and pGAD were kindly supplied by Dr. S. Elledge (Baylor College of Medicine). Parental expression vectors for mammalian 2-hybrid analysis, pSG424 and pNLVP16, were obtained from Dres. C. Dang (Johns Hopkins University School of Medicine) and M. Green (University of Massachusetts) (45, 46). Murine Id1 was obtained from Dr. H. Weintraub (Fred Hutchinson Cancer Research Center), and murine Id2 and Id3 cDNAs were obtained from Dr. D. Nathans (Johns Hopkins University) (28, 30, 34). Fragments encoding the HLH regions plus 15–20 flanking amino acid residues were generated by PCR primers incorporating EcoRI (forward primer) and BamHI (reverse primer) sites and cloned directionally and in-frame into pGAD, pGBT9, and pSG424. The fragments amplified encoded amino acids 73–138 of Id1, 72–140 of Id2, and 28–91 of Id3. All products were sequenced to confirm the fidelity of the PCR amplification reaction. Full-length cDNAs were cloned into pRCMV (Invitrogen, San Diego, CA) for use in in vitro transcription/translation reactions and transient transfection assays. Murine MyoD, murine myogenin, and human Myf-5 were supplied by Dr. D. Shapiro (St. Jude Children’s Research Hospital) and rat MRF4/Myf-6 by Dr. S. Konieczny (Purdue University) (8, 15, 16, 20). Fragments encoding the bHLH domains were again amplified by PCR, incorporating EcoRI and BamHI sites for MyoD or BamHI sites for Myf-5 and Myf-6 and cloned in-frame into pGBT9 and pGAD424. The regions amplified included codons 83–184 of MyoD, 55–155 of myogenin, 56–130 of Myf-5, and 65–183 of MRF4/Myf-6. Due to the presence of an internal PstI site in the second helix of Myf-5 and MRF4/Myf-6, these were cloned as BamHI/blunt fragments. The bHLH domains from MyoD and Myf-5 were also subcloned in-frame into pNLVP16 as blunt-ended fragments. Full-length MyoD and MRF4/Myf-6 cDNAs were cloned into pRCMV. The cDNAs encoding each of the class A factors were isolated from a yeast 2-hybrid screen of a murine embryo library in the pGAD10 vector (CLONTECH, Palo Alto, CA) and subcloned into Id2 bait vector. These contained residues 118–264 of murine E47 (A1), residues 379–666 of murine E2-2 (ME2), and residues 574–729 of murine HEB (A1F-1) (47–49). The E47 fragment was also cloned into both pNLVP16 and pGBT9. A carboxy-terminal bHLH containing fragment of human E12 (residues 508–654) in the pAS1 vector, a gift from Dr. E. Olsen (University of Texas), was excised and sub-cloned into pGAD424 (44).

None of the class A clones contained the putative leucine zipper regions that are associated with transcriptional activation domains (50). An almost full-length E12 clone in blue script (E12R, a gift from Dr. H. Weintraub), including the putative leucine zipper domain, was used for in vitro translation (6). Full-length E12 and E47 in the mammalian expression vector pGK W12 were provided by Dr. G. Kado (University of Texas Southwestern Medical School) and M. Green (University of Massachusetts) (51). The muscle creatinine kinase (MCK) CAT construct was kindly supplied by Dr. S. Elledge (Baylor College of Medicine). Parental expression vectors for mammalian 2-hybrid analysis, pSG424 and pGBK7 were again amplified by PCR with primers containing EcoRI and BamHI sites and were cloned in-frame into both pGAD424 and pGBT9. The amplified fragments contained amino acids 66–138 of Sc/Tal-1, 2–69 of Tal-2, and 127–200 of Lyl-1. Full-length Sc/Tal-1 was subcloned into pBluescript SK + (Stratagene, La Jolla, CA). The multimerized gal4::CAT reporter construct pGal5E472CAT was supplied by Dr. M. Green (51). The muscle creatinine kinase (MCK) CAT construct was obtained from Dr. S. Hauenschka (University of Washington).

**Yeast 2-Hybrid System**—This was performed according to the protocol developed in the laboratory of Dr. S. Elledge, who also supplied the Saccharomyces cerevisiae strain Y153 (MATa, leu2-3, trp1-901, his3-d200, ura3-52, ade2-101, gal4A, gal80GAL-HIS3, LYS2::GAL-HIS3). Potential partner genes in either pGAD424 or pGBT9 were co-transformed into yeast by a lithium chloride/polyethylene glycol precipitation method (52). Semi-quantitative determination of interaction strengths was performed by in situ quantitation of β-galactosidase activity.

Quantitative β-Galactosidase Assay—Yeast transformants were grown to stationary phase in complete EGG medium containing 2% ethanol, 2% galactose, and 3% glycerol and lacking tryptophan and leucine. 10^6 cells were pelleted and resuspended in 50 μl of Z buffer (60 mm Na2HPO4, 40 mm NaH2PO4, 10 mm MgCl2, 50 mm β-mercaptoethanol) containing 0.01% SDS. Two microliters of CHCl3 were added followed by two cycles of freeze-thawing in liquid nitrogen. Lysates were transferred to 96-well plates, and 50 μl of a fluorogenic substrate (8 mM 3-carboxyumbelliferyl-β-D-galactopyranoside, Molecular Probes Inc., Eugene, OR) was added. After 30 min, 100 μl of stop buffer (300 mm glycine, 15 mm EDTA, pH 11.5) was added, and the reactions were allowed to stabilize for 1 h. Fluorescence was determined in a Perkin–Elmer microtiter plate reader (Foster City, CA; excitation 390 nm, emission 460 nm), and the amount of β-galactosidase synthesized was calculated relative to a dilution series of β-galactosidase enzyme standards (Sigma) assayed simultaneously with the yeast lysates. All assays were performed in triplicate with standard errors of less than 10% in each case. None of the individual vectors used in this series displayed a background higher than 10% of β-galactosidase synthesized by yeast cells which represents the lower limit of detection with this assay.

**Western Blot Analysis**—Yeast were grown in EGG medium until they reached stationary phase. They were then pelleted and subject to two cycles of freeze-thawing followed by boiling in standard SDS-polyacrylamide gel electrophoresis (PAGE) lysis buffer for 10 min to ensure complete lysis (53). 100 μg of protein was resolved by 12% PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA) in the presence of 1 mol/L glycine, 2 mol/L ethanol, 2% galactose, and 3% glycerol and lacking tryptophan and leucine. Membranes were preincubated in wash solution (10 mm Tris, 1 mm EDTA, pH 7.4) for 1 h and then washed four times in PBS-Tween 20 (0.1% Tween 20) containing either an anti-yeast gal4 DNA binding domain or activation domain antibody (at 1:500 and 1:1,000 dilutions, respectively) (Upstate Biotechnology Inc., Lake Placid, NY). Membranes were washed four times before the addition of a horseradish peroxidase-linked goat anti-mouse antibody (1:100 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA; excitation 320 nm, emission 460 nm), and the amount of β-galactosidase synthesized was calculated relative to a dilution series of β-galactosidase enzyme standards (Sigma) assayed simultaneously with the yeast lysates. All assays were performed in triplicate with standard errors of less than 10% in each case.

**In Vitro Transcription/Translation and Co-immunoprecipitation**—Full-length E12, Id1, Id2, Id3, Sc/Tal-1, MyoD, and MRF4/Myf-6 cDNAs in either pBlueScript SK+ or pRCMV were transcribed and translated in vitro using a coupled reticulocyte lysate kit (TNT, Promega, Madison, WI) in the presence of [35S]methionine (1 μCi/ml). Labeled proteins were monitored and quantitated at 1 h before the addition of 100 μl of IP buffer (250 mm NaCl, 0.5% Nonidet P-40, 20 mm Tris-HCl, pH 7.5, 1 mm EDTA, 1 mm dithiothreitol). Anti-Id, E12, or MdyoD polyclonal antibodies were added and reactions incubated on ice for 30 min prior to the addition of 30 μl of a 1:1 mix of protein A-Sepharose (Bio-Rad) in IP buffer. After a further 60 min incubation, samples were washed four times in IP buffer before resolution by 12% SDS-PAGE. The intensity of each partner was quantified by Phosphor-
Mashalov and colleagues investigated the interactions between Id and MyoD transcription factors. They used a quantitative yeast 2-hybrid approach to determine the interactions between Id proteins and the myogenic factors MyoD and Myf-5. The results showed that Id1 bound both MyoD and Myf-5 with a 5–10-fold greater affinity than Id3, which demonstrated barely detectable interactions. Id2 displayed the strongest binding which, in the case of MyoD and Myf-5, was 3–4-fold more avid than that of the corresponding Id1 interactions. In contrast to those seen between the Ids and the class B myogenic factors (E2A and E47), all the myogenic factors (including MRF4/Myf-6) interacted strongly (Fig. 1). Hence the differences in binding seen here cannot be attributed to differential expression. This was also confirmed by Western blot analysis of yeast lysates showing comparable levels of expression of all proteins under study (Fig. 2). When tested for their ability to bind the class A factors E47 or E12, all the myogenic factors (including MRF4/Myf-6) interacted strongly (Fig. 1B), again indicating that the differences in myogenic factor-Id binding were not due to relative differences in MRF protein expression. None of the hematopoietic factors displayed discernible interactions with the Id proteins, whereas all were found to interact with the class A target E2-2 (Fig. 1C).
MRFs were also investigated for homodimerization. MyoD, myogenin, and Myf-5 displayed weak homodimerization (less than 50 pg of β-galactosidase detected), whereas MRF4/Myf-6 homodimerization was not observed. Neither the Ids nor the hematopoietic factors displayed discernible homodimerization ability in this assay (data not shown).

Co-immunoprecipitation Analysis—Co-IP of selected full-length in vitro translated proteins was employed to confirm the differences in dimerization properties identified by yeast 2-hybrid analysis (Fig. 3). Increasing amounts of Id1, Id2, or Id3 proteins were incubated with E12, and resultant heterodimers were precipitated with an anti-E12 polyclonal antibody (Fig. 3A). Consistent with the yeast 2-hybrid data (Fig. 1), all three Ids interacted strongly with E12 with any differences being on the order of less than 2-fold. In contrast, interactions between Id proteins and MyoD were considerably weaker and showed more pronounced differences than those observed with Id-E protein interactions. For example, Id1 and Id2 showed comparable interactions with MyoD, whereas Id3-MyoD interactions were significantly weaker. These results were in good agreement with the results obtained by the 2-hybrid system. All Ids also bound very weakly to MRF4/Myf-6 (Fig. 3C) and not at all to Scl/Tal-1 (Fig. 3D). From these experiments, we conclude that the strengths of Id-E protein and Id-MRF interactions qualitatively and quantitatively reflected those observed in yeast. Furthermore, our results indicate that the dimerization properties of full-length proteins are not significantly different from those of the isolated HLH domains expressed in yeast.

Mammalian 2-Hybrid Analysis—The observation that the Ids displayed the widest range of interactions with MyoD and Myf-5 led us to investigate whether such differences were maintained in mammalian cells (Fig. 4). Id1, Id2, and Id3 were expressed as VP16 transactivation domain fusions, whereas MyoD and Myf-5 were expressed as VP16 transcription domain fusions (56). Each was tested for its ability to activate a reporter vector in HeLa cells (Fig. 4A). E47, which displays strong interactions with each Id in yeast (Fig. 1A), was also expressed as a VP16 fusion as a positive control for Id dimerization activities. Appreciable CAT conversion was seen when either Id1 or Id2 was co-expressed with either MyoD or Myf-5 (Fig. 4B), whereas Id3 interaction with either of the myogenic factors was only marginally above background. These observations were generally consistent with both the yeast 2-hybrid data and with the co-IP experiments (Figs. 1 and 3). As the Ids differed in their ability to dimerize with the MRFs, we investigated their ability to bind E47 (Fig. 4B). Each was found to generate comparable high levels of CAT activity as might have been predicted from our yeast 2-hybrid results (Fig. 1A). Thus the reduced ability of Id3 to interact with MyoD or Myf-5 cannot simply be explained by its differential expression. Our results in both mammalian cells and in yeast appear to reflect the true dimerization preferences of the Id proteins under study.

Id Repression of E Protein-MyoD Heterodimer Activity—By having established that the Ids interacted similarly with each E protein (Fig. 1A), we sought to determine the influence of each Id on the transcriptional activity of full-length E2A-MyoD heterodimers (Fig. 5). We had previously established that this combination of factors gave the highest levels of transactivation from the MCK-CAT reporter. The relative consistency of Id-E2A interactions in yeast suggested that each Id should disrupt E2A-MyoD complexes with comparable ability. Low levels of transactivation were observed when either 5 μg of MyoD or 1 μg of E47 or E12 was transfected alone. Co-transfection of these factors, however, generated significant CAT conversion, indicating that transactivation was mediated by an E2A-MyoD heterodimer and not by endogenous factors. The enhanced ability of MyoD to form heterodimers with E47 compared with E12 in yeast (Fig. 1B) was reflected in an increased ability of MyoD-E47 heterodimers to activate the MCK-CAT reporter. The introduction of increasing amounts of each Id led to the suppression of CAT activity in a dose-dependent fashion.

2 K. Langlands, X. Yin, G. Anand, and E. V. Prochownik, unpublished data.
Id-bHLH Interactions

with a maximal 2–3-fold reduction in transactivation observed at the highest input Id concentration. In metabolic labeling experiments, the expression of each Id was determined to be comparable (data not shown).

Id Protein Helix Swaps and Site-directed Mutagenesis—To map the amino acid residues responsible for the differential binding capabilities of Id proteins with respect to the MRFs, helix swaps were generated, and site-directed mutagenesis was performed (Fig. 6). All mutants were tested in yeast for their ability to bind E12, and all were found to bind with similar avidity, varying over an approximate 2-fold range (Fig. 6, last column). These results were anticipated based on our observations that wild-type Id1 and Id3 interacted to a comparable extent with E12 (Fig. 1A). Initially, chimeras containing the first helix of Id1 and the loop-helix 2 region of Id3 or the first helix of Id3 and the loop-helix 2 region of Id1 were constructed and tested for interaction with all four MRFs (Fig. 6, lines 3 and 4). These experiments clearly demonstrated that the region determining the specificity of interactions resided in the first helix and/or in the amino-terminal region immediately adjacent to this. Based on this observation, the three amino acid residues that distinguish the first helices of Id1 and Id3 were altered sequentially to convert Id1 to Id3 and vice versa. Altering individual tyrosine, glycine, and lysine residues of Id1 to the corresponding aspartic acid, histidine, and arginine residues of Id3, respectively, reduced binding in each case (Fig. 6, lines 5, 7, and 9). However, the relative importance of each residue for dimerization was dependent upon the MRF under study. For example Id1/3 (G92H) showed a 3-fold reduction in the ability to bind MyoD but a 14-fold reduction in the ability to bind Myf-5 (compare Fig. 6, lines 5 and 7). In contrast Id1/3 (K98R) showed a 12-fold reduction in MyoD binding but only a 4-fold reduction in myogenin binding (compare Fig. 6, lines 3 and 9). Similarly, no single Id3 to Id1 mutation restored maximal binding (Fig. 6, lines 6, 8, and 10). This demonstrates that each residue, even the conserved lysine of Id1 and arginine of Id3, contributes to binding specificity although the relative importance of each residue is dependent upon the MRF target. To investigate possible additive effects, two further sets of Id3 mutations were investigated for their ability to restore Id1-like binding. A double mutant (Fig. 6, line 11), created by altering the aspartic acid and histidine residues of Id3/1 to the complementary tyrosine and glycine residues of Id1, resulted in an almost full binding to MyoD, increased binding to Myf-5, and weak binding to MRF4/Myf-6. This double mutant did not, however, restore myogenin binding. Even when all three residues were altered to those of Id1 (Fig. 6, line 12), no increase was seen in Myf-5 binding when compared with the double mutant. Some binding was seen to myogenin, whereas MRF4/Myf-6 binding was undetectable. These data suggested that helix 1 residues are sufficient for MyoD recognition but that additional residues upstream of helix 1 appear to be required for full dimerization with the other three myogenic factors. To investigate the upstream requirement further, a series of deletions were made in the Id1/3 swap background. The initial deletion of six amino acids at the extreme NH2 terminus of the Id1/3 sequence (Fig. 6, line 13) had a minimal effect on binding (<2-fold) with the exception of Myf-5 whose binding was reduced approximately 3-fold (Fig. 6, lines 3 and 13). The deletion of a further six residues (Fig. 6, line 14) resulted in the abrogation of myogenin and MRF4/Myf-6 binding although little

**Fig. 4. Mammalian 2-hybrid analysis.** A, an interaction between an MRF (expressed in pNLVP16) and an Id protein (expressed in pSG424) will result in the activation of a CAT reporter gene bearing an MRF (expressed in pNLVP16) and an Id protein (expressed in pSG424) will result in the activation of a CAT reporter gene bearer. B, HLH domains of Id1, Id2, and Id3, expressed as gal4 DNA binding domain (BD) fusions, were tested for their ability to interact with MyoD, Myf-5, or E47 expressed as VP16 transactivation domain (TAD) fusions. CAT conversion over background was determined by PhosphorImage analysis. Values shown represent the average of three independent experiments with standard errors of less than 10%.
additional effect was seen with respect to Myf-5 and MyoD.
Finally, an internal deletion of the six residues immediately
adjacent to the amino terminus of the first helix (Fig. 6, line 15)
severely inhibited dimerization with all MRFs, although this
was least obvious with MyoD. Interestingly, all of the individ-
ual deletion mutants (Fig. 6, lines 13–15) bound E12 as well as
either of the wild-type Ids (with <2-fold differences). From
these studies, it appears that complete MyoD and Myf-5 inter-
actions with Id1 require similar amino acid residues. The res-
idues facilitating MyoD and Myf-5 binding are not sufficient for
myogenin and MRF4/Myf-6 binding, and these latter factors
require additional, upstream amino acids. Binding by E12
would appear not to require any of these upstream residues.

A select number of the Id "helix swap" proteins depicted in
Fig. 4 were examined using the mammalian two-hybrid sys-
tem. In these experiments, the HLH domains of Id1/3, Id3/1,
and Id1/3 D80–85 were cloned into the pSG424 vector and
transfected into HeLa cells together with either pNLVPMyoD
or pNLVPMyl5. As shown in Fig. 7, these results recapitulated
those observed in yeast. Neither Id3, Id3/1, nor Id1/3 D80–85
gave a detectable interaction with either MyoD or Myf5. In
contrast, Id1 interacted strongly. That each of the Id proteins
was expressed was confirmed in control experiments showing
that all five Id fusion proteins depicted in Fig. 7 interacted
strongly with a pNLVP16 fusion of the E12 bHLH domain (not
shown).

**DISCUSSION**

bHLH proteins are involved in diverse aspects of cellular
physiology, from establishing the topography of the early em-

**Fig. 6. Id protein helix swaps and site-directed mutagenesis.** A series of Id hybrid molecules were tested in their ability to bind all four
MRFs and E12 using a quantitative yeast 2-hybrid assay, performed in triplicate. The amount of β-galactosidase enzyme generated per 10^7 cells (ng) for each set of interactions is shown in the right hand columns. Standard errors were less than 5% in each case.
by co-IP and glutathione S-transferase-pull down analysis (28,
34, 41). Mammalian 2-hybrid approaches have also dem-
strated the ability of Id1 to interact with both MyoD and E12
and of Scl/Tal-1 to interact with E47 (56, 64). These reports
concur with the general observation that class A-class B
and Id-class A complexes are more stable than Id-class B com-
plexes. Our study not only confirms and extends previous re-
ports of HLH protein dimerization potentials but attempts to
provide a consistent comparison of a broad range of
interactions.

All three Id proteins tested here interacted strongly with all
class A factors, consistent with their role in the sequestra-
tion of these ubiquitous proteins. Although Id-class A interac-
tions displayed the strongest and most consistent interactions
among the series of factors tested, each Id nevertheless dis-
played a discrete "fingerprint" of preferred class A partner.
Specific patterns of Id-MRF interactions were also observed,
although these interactions were considerably weaker and
showed greater variability than those of Id-class B com-
plexes. The finding that Id-MRF interactions were conserved, in con-
trast to Id-hematopoietic factor interactions, suggests that the
Ids may have a role in myogenesis that is distinct from that in
hematopoiesis. Finally, the overlap in Id-class B and Id-class A
interaction strengths provides additional reason to believe that
the former may be physiologically relevant. When the Ids were
expressed as full-length proteins in vitro, or as HLH domains in
human cells, the differences in relative affinities observed in
yeast persisted (Figs. 3 and 4), indicating that the discrete
range of interactions accurately reflects the in vivo situation
and was not merely a consequence of the expression of mam-
malian genes in yeast.

Homodimerization of bHLH proteins seems to be important
in certain contexts. For example, E47 homodimers are suf-
cient to activate immunoglobulin gene expression (65). MyoD is
also capable of binding DNA as a homodimer, although it has
not been established if this is biologically significant as the
concentrations required for homodimerization in vitro may not
be achievable in vivo (8). On the other hand, it is conceivable
that apparently weak interactions are stabilized by accessory
factors or that low concentrations of MyoD homodimers are
sufficient to initiate the myogenic cascade, and this may be
sensitive to direct Id-mediated suppression. Therefore, cell fate
determination may in part depend on the relative level of
distinct Id proteins to create a permissive environment. Inter-
estingly, our observations indicate that the Ids bind with the
highest affinity those myogenic factors involved in myoblast
lineage determination (MyoD and Myf-5), whereas those MRFs
active post-mitotically (myogenin and MRF4/Myf-6), a time
when Id levels are generally low or undetectable, bind Ids
considerably less well.

The differential affinities of the Id proteins for both the class
A and class B MRFs could be explained if these latter proteins
homodimerized. Under such conditions, there might exist a
differential availability of E proteins or MRFs for Ids due to
their sequestration in homodimeric form. In other experiments,
we have examined the ability of the Ids, the E proteins, and the
class B MRFs and hematopoietic factors to homodimerize using
the same two-hybrid system shown in Fig. 1. We detected
homodimerization only with E47, although at levels that were
10–30-fold lower than its interaction with any of the three Ids.
Thus, limitation of E protein accessibility to the Ids due to
homodimeric sequestration appears unable to explain our
results.

Having demonstrated that Id proteins bind each class A
molecule and a subset of class B molecules, we tested the

\[ K. \text{ Langlands and E. V. Prochownik, unpublished data.} \]
ability of each Id to suppress transactivation from a muscle-specific E box by an E2A-MyoD complex in vivo (Fig. 5). Id1, Id2, and Id3 were found to disrupt E2A-MyoD complexes with comparable ability, consistent with the yeast data. Interestingly, an apparent excess of Id was unable to completely abrogate binding. Other studies on the effect of Id1 or Id3 on the transactivation potential of MyoD or E proteins have also reported a less than complete suppression of activity, with even greater excesses of Id than we have utilized (28, 35). Investigation of the activity of any transcription factor in isolation is complicated by the influence of endogenous factors. Having established conditions in which each factor alone results in minimal transactivation, we can be certain that the repressive effect of the Ids is exerted only on the factors under study.

The observation that the Ids differed in their ability to recognize myogenic partners led us to investigate the precise residues responsible for this specificity (Fig. 6). Dimeric bHLH complexes bound to DNA are predicted to form a parallel 4-helix bundle stabilized by a hydrophobic core as well as by electrostatic interactions (2, 8, 62). Shirakata et al. (62) reported that the alteration of five non-hydrophobic residues distinguishing chicken MyoD from the Drosophila MyoD homologue nautilus (which does not bind E12) led to a progressive reduction in MyoD/E12 dimer formation, suggesting that these residues confer an additive effect on binding (62). Likewise, in our study, no single residue was able to confer Id1-like characteristics. Rather a combination of non-conserved residues was involved in MRF binding. However, these residues did not appear to be important for Id-class A interaction (Fig. 6). Two non-conserved residues in the first helix were sufficient for almost complete MyoD binding. These residues, Tyr-88 and Gly-92 of Id1, are uncharged, whereas the corresponding residues of Id3 (Asp-42 and His-46) confer a negative and positive charge, respectively. Thus, the differences in Id binding (with respect to MyoD) may be consistent with the “charged pair” model which suggests that attractive or repulsive forces between contacting residues in the helices of aligned molecules act to stabilize or destabilize dimer formation (62). The residue at the third position also influences heterodimerization even though this represents a conservative change (Lys-98 in Id1 to Arg-52 in Id3). In this case, the larger arginine side chain may act to destabilize Id3-MRF complexes. Despite the obvious importance of these residues, the complete conversion of Id3 helix 1 to that of Id1 helix 1 restored only 50% Myf-5 binding and only minimal interaction with myogenin and MRF4/Myf-6, suggesting that additional residues exert an effect. Indeed, a series of amino-terminal deletions (Fig. 6, lines 13 and 14) demonstrated that a region outside the HLH domain was important for Id1-MRF interactions, particularly with respect to myogenin and MRF4/Myf-6. Interestingly, these deletions did not appear to influence E12 binding. Upstream sequences may not be involved in establishing dimeric complexes as such, but rather in stabilizing pre-formed interactions determined by residues in the HLH region. Such subtle cooperative effects might be expected to contribute more to weak Id-myogenin or Id-MRF4/Myf-6 interactions than to stronger associations.

Goldfarb et al. (43) used random mutagenesis in conjunction with a yeast 2-hybrid system to generate both hydrophobic and hydrophilic substitutions which enhanced the ability of Scl/ Tal-1 to recognize E2-2 (43). Such alterations were again found to have a synergistic effect on dimer stability. More extensive mutagenesis is required to fully understand the diversity of dimer preferences displayed by HLH factors. The quantitative yeast 2-hybrid system described in this report provides a useful tool for this purpose.

Regions other than the HLH domain, such as the putative leucine zipper present in the class A proteins, have the potential to modulate interactions (1, 11, 12). Because our co-IP findings with full-length proteins are consistent with both our yeast and mammalian 2-hybrid results, we infer that other potential dimerization domains do not contribute significantly to the interactions observed here. However, we have not evaluated the possible influence of post-translational modifications on dimerization (47, 59, 66, 67).

Although HLH activity may be modulated in vivo, the key differences in binding characteristics presented in this study may play a fundamental role in the establishment of differentiation programs. There still exists, however, a great deal of redundancy. One apparent paradox is that there is little evidence, as yet, for the combinatorial variation in E box binding allowed by heterodimerization between different class B groups and any class A factor. For example, all class A-MRF dimers so far studied efficiently bind an element in the muscle creatine kinase enhancer, and binding site selection indicates that Scl/ Tal-1, Tal-2 and Lyl-1 heterodimers with E2-2 all recognize the same putative hematopoietic-specific element (68, 69). Spatial and temporal differences in the expression of factors with apparently conserved function may also explain some of the apparent overlap. Alternatively, redundancy may reflect the need for cooperative occupancy of proximal promoter elements by accessory factors. A distinct group of tissue-restricted non-bHLH transcription factors have been identified, such as the myocyte enhancer family (MEF-2) in muscle and the zinc finger GATA factors in erythropoiesis (70, 71). These may act to enhance the transcriptional activity of bHLH dimers, thereby modulating lineage-specific gene expression. Indeed, the apparent redundancy in function may mask the requirement for a subtle gradient of both ubiquitous and tissue-specific factors to facilitate the controlled establishment of the differentiated phenotype.

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