Enhancer-specific Modulation of E Protein Activity*

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Homodimeric complexes of members of the E protein family of basic helix-loop-helix (bHLH) transcription factors are important for tissue-specific activation of genes in B lymphocytes (Bain, G., Gruenwald, S., and Murre, C. (1993) Mol. Cell Biol. 13, 3522–3529; Shen, C. P., and Kadesch, T. (1995) Mol. Cell Biol. 15, 4518–4524; Jacobs, Y., et al. (1994) Mol. Cell Biol. 14, 4087–4096; Wilson, R. B., et al. (1991) Mol. Cell Biol. 11, 6185–6191). These homodimers, however, have little activity on myogenic enhancers (Weintraub, H., Genetta, T., and Kadesch, T. (1994) Genes Dev. 8, 2209–2211). We report here the identification of a novel cis-acting transcriptional repression domain in the E protein family of bHLH transcription factors. This domain, the Rep domain, is present in each of the known vertebrate E proteins. Extensive mapping analysis demonstrates that this domain is an acidic region of 30 amino acids with a predicted loop structure. Fusion studies indicate that the Rep domain can repress both of the E protein transactivation domains (AD1 and AD2). Physiologically, the Rep domain plays a key role in maintaining E protein homodimers in an inactive state on myogenic enhancers. In addition, we demonstrate that Rep domain mediated repression of AD1 is a necessary for the function of MyoD-E protein heterodimeric complexes. These studies demonstrate that the Rep domain is important for modulating the transcriptional activity of E proteins and provide key insights into both the selectivity and mechanism of action of E protein containing bHLH protein complexes.

The basic helix-loop-helix (bHLH) family of transcription factors plays an important role in embryonic patterning, cell fate determination, cellular differentiation, and proliferation decisions (reviewed in Ref. 1). Structurally, the bHLH domain is a 60-amino acid region containing two helices separated by a loop segment preceded by a region rich in basic residues. The basic region is responsible for DNA binding and the HLH region is important for dimerization with other members of the family (2–4). The bHLH family has been divided into three major classes. Class I consists of the ubiquitously expressed E proteins. There are three E protein family members in mammals, E2A (with three major splice products: E12, E47, and E2-5), E2-2 (also called ITP2) and HEB (2, 5–9). Class II bHLH proteins, such as MyoD, are expressed in a tissue-specific manner (10) and require E proteins as obligate heterodimeric partners for DNA binding and transcriptional activation (11). Cell fate decisions are often regulated by alternations in the expression of these tissue-specific proteins. Finally, class III members include the dominant negative Id proteins that heterodimerize with the E proteins but, because they lack a basic domain, form heterodimeric complexes incapable of binding DNA (12, 13).

In addition to their role as heterodimeric partners for tissue-specific bHLH proteins, E proteins are also capable of forming homodimeric complexes, which can activate a variety of B cell-specific genes and are necessary for B cell differentiation (14–20). The E proteins have two transcriptional activation domains, activation domain 1 (AD1) and 2 (AD2) (21–23). AD1 is contained within the first 100 amino acids of the protein and contains a putative α-helix. AD2, also termed the loop-helix domain, is located midway between the AD1 and bHLH domains. The transcriptional activation domains of the E proteins play a critical role in cellular transformation in the context of chimeric oncoproteins such as E2A-HLF and E2A-Pbx1 (24–26).

Site selection studies have suggested that enhancer selection by specific bHLH complexes is to some extent regulated by the sequences within and surrounding the E box (27). Binding site selection is not sufficient, however, to explain the specificity of action of many bHLH proteins. It has been demonstrated that the myogenic bHLH protein MyoD can bind to the B cell-specific immunoglobulin enhancer, but is transcriptionally inactive as a result of a cis-acting repressive element in the enhancer (28). Similarly, E protein homodimers can bind to E boxes within myogenic enhancers, but these complexes are almost entirely inactive (28). The mode of repression of these E protein homodimers is unknown.

In this study, we have identified a novel autoregulatory domain common to all of the vertebrate E proteins. This domain, which we term the Rep domain, is a potent inhibitor of the AD1 and AD2 transcriptional activation domains. We demonstrate that the Rep domain plays a key role in inactivating E protein homodimers bound to a myogenic enhancer. In addition, this domain is required to form active MyoD-E protein heterodimeric complexes by repressing AD1, which is inhibitory on the MCK enhancer. Thus, the Rep domain modulates the activity of E protein-containing bHLH protein complexes and may play a key role in regulating the activity of complexes bound to tissue-specific enhancers.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Full-length E2-2 and E12 were amplified by PCR and cloned into a pcDNAIII vector (Invitrogen). All GAL4 DNA binding domain fusions were constructed by ligation of E protein DNA in-frame into a modified pM3 vector (1). Deletions of E12 and E2-2 were generated by use of appropriate restriction enzyme sites and by PCR. E12ΔAD1 ΔAD2 and E12Δrep ΔAD1 ΔAD2 were cloned into pFLAG CMV2 vector (Kodak). All constructs were verified by sequencing.
EMC11S is the expression vector for MyoD (29). Reporter vectors used were: multimerized E box driving chloramphenicol acetyltransferase (CAT), 4RtkCAT (30); multimerized GAL4 binding sites driving luciferase, GAL4-luc (31); muscle creatine kinase enhancer driving luciferase, MCK luciferase; multimerized immunoglobulin enhancer elements driving luciferase, (mElmE5)6 luciferase (gift of T. Grundstrom).

Site-directed mutagenesis of the Rep domain was performed with a Site-Directed Mutagenesis kit as per the manufacturer’s instructions (Strategene). The following oligonucleotides were used for mutagenesis: 5′-GCGGCCTGGCTCCCGAAATCCGGATGAGC-3′ (mutant 1); 5′-GCGGCCTGGCTCCCGAAATCCGGATGAGC-3′ (mutant 2); 5′-CGTTTATTACGGTGCAGAGGTCATGAGGACAG-3′ (mutant 3); 5′-GATCAAATCCGGATGAGC-3′ (mutant 4); 5′-CGAATCTCCGGCTCCCGAAATCCGGATGAGC-3′ (mutant 5); 5′-GACGAGGTTGATGAGCGGCGGACAAGCAGGAATC-3′ (mutant 6); 5′-GCTGATGAGCAACCTGGCAGCCAAGAATCCGGATGAGC-3′ (mutant 7); 5′-GAGAACCTGGACAAGCGGCGGACAAGCAGGAATC-3′ (mutant 8); 5′-CGGAGAACCTGGACAAGCGGCGGACAAGCAGGAATC-3′ (mutant 9); 5′-CGGAGAACCTGGACAAGCGGCGGACAAGCAGGAATC-3′ (mutant 10); 5′-GCGAGAACCTGGACAAGCGGCGGACAAGCAGGAATC-3′ (mutant 11); 5′-CGGAGAACCTGGACAAGCGGCGGACAAGCAGGAATC-3′ (mutant 12); 5′-GAGAACCTGGACAAGCGGCGGACAAGCAGGAATC-3′ (mutant 13); 5′-GAAATACGTGCAGGCGGACAAGCAGGAATC-3′ (mutant 14). Mismatched nucleotides are indicated in boldface. All constructs described above were verified by DNA sequencing.

Cell Culture and Transfections—HeLa, C3H10T1/2, and COS-7 cells were maintained in Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) (HyClone) and plated at 50% confluence. COS-7 cells were plated at a 50% confluence and transfected with 3 mg of expression plasmid 12–24 h later by the calcium phosphate method. Nuclear extracts were prepared after 48 h according to the procedure of Schreiber et al. (33). 60 mg of protein was fractionated on a 10% discontinuous SDS gel and electrotransferred to a nitrocellulose filter. After transfer, the filter was incubated for 30 min at room temperature in 5% milk. Primary antibody incubation was carried out at room temperature for 1 h in PBS containing 0.05% Tween 20 and 200 ng/ml anti-GAL4 DBD antibody (sc-577, Santa Cruz Biotechnologies) for 1 h. After primary antibody binding, the filter was washed three times with PBS containing 0.05% Tween 20. Secondary antibody incubation was carried out at room temperature for 1 h in PBS containing 0.05% Tween 20 and donkey anti-rabbit immunoglobulin conjugated to horseradish peroxidase diluted 1:10,000. The filter was then washed five times with PBS containing 0.05% Tween 20, and proteins were detected by chemiluminescence (Amersham Biosciences, Inc.).

All mobility shift assays were performed as described previously (12). 6 mg of soluble nuclear protein was incubated with probe DNA for 5 min at room temperature. Supershifts were performed by addition of 1 mg of anti-FLAG M2 monoclonal antibody (Sigma Chemical Co.) to the binding reaction prior to gel loading. The DNA binding construct contained 200 bp HEPES (pH 7.6), 50 mM KCl, 1 mM EDTA, 6% glycerol, and 100 μg/ml poly(dI-dC). The upper strand of the oligonucleotides used as probes were as follows: E box (sense), 5′-CCCCACACCTGGCTCCGGACAAGAATCCGGATGAGC-3′; Gal binding site (sense), 5′-GATCGCCGAGTACTGGCTCCGGACAAG-3′.

Immunofluorescence Assay—C3H10T1/2 cells transfected with expression plasmids were fixed in 2% paraformaldehyde for 30 min at room temperature and washed three times in PBS. Slides were blocked with MOM™ Kit (Vector Laboratories) blocking reagent (Zymed Laboratories Inc.), incubated with anti-myocin-heavy-chain antibody (Zymed Laboratories Inc.) overnight at 4 °C, then incubated 1 h with anti-mouse IgG Texas Red-conjugated antibody (Jackson ImmunoResearch) at room temperature. After three final washes in PBS containing 5% bovine serum albumin and 0.5% IGEPAL, slides were mounted with SlowFade mounting reagent (Molecular Probe).

RESULTS

Deletions in E2-2 Reveal a Transcriptional Repression Domain—In a screen to map functional domains in E2-2, sequences in and around activation domain 2 (AD2) (226–495) of E2-2 were fused to the DNA binding domain of GAL4. These expression plasmids were cotransfected into HeLa cells with a luciferase reporter plasmid containing five GAL4 DNA binding sites, and luciferase activity was quantitated after 24 h. A GAL-E2-2 fusion that spans residues 226–495 is a very poor transcriptional activator (Fig. 1) whereas a shorter GAL-E2-2 fusion containing amino acids 226–493 is 82.3-fold more active. This suggested that E2-2 contains a domain capable of partially inactivating AD2. To test whether E12 (a product of the E2A gene) also contains this domain, the GAL4 fusion domain studies were repeated with E12 (Fig. 1). A fusion of amino acids 230–524, containing AD2 and significant C-termi-
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To confirm that repression is not due to an alteration in the DNA binding capacity of the E2-2 constructs, nuclear extracts from transfected cells were incubated with a radiolabeled E-box containing oligonucleotide, and protein-DNA complexes were resolved by non-denaturing gel electrophoresis (Fig. 2E). An equivalent concentration of E box binding activity was present in cells transfected with the various E2-2 constructs. These data demonstrated that the transcriptional repression mechanism is unrelated to alterations in the dimerization or DNA binding capacity of E2-2.

The Repression Domain Is Sufficient to Inhibit AD1 and AD2 and Is Common to All of the Vertebrate E Proteins—The minimal E2-2 Rep domain was fused to AD1 and AD2 of E2-2 and to the activation domain of the viral transcription activator VP16. Each of these chimeric proteins was expressed as a fusion with the GAL4 DNA binding domain. As shown in Fig. 3A, fusion of the Rep domain onto AD1 (top panel) and AD2 (middle panel) repressed transactivation to nearly background levels indicating that the Rep domain is sufficient for transcriptional inhibition. Fusion of the Rep domain onto the VP16 activation domain, however, had no significant effect on transcription, indicating at least some degree of specificity for E protein activation domains. We observed, however, that the Rep domain cannot inhibit E protein activation domains when bound separately to an adjacent DNA element (data not shown), suggesting that the Rep domain may function exclusively as an intramolecular transcriptional repressor.

Examination of the Rep domain sequence reveals a region rich in polar and charged amino acids (particularly lysine, aspartic, and glutamic acid) that is remarkably conserved within the E protein family in all characterized vertebrate E proteins (Fig. 3B). Overall, the Rep domain of human E2-2 and Zebrafish E12 are 43% identical. PHD (Profile network prediction HeiDelberg) analysis predicted this stretch of amino acids to adopt a flexible loop structure.

To assess the contribution of each amino acid residue to the Rep domain function, we performed alanine scan mutagenesis. Each residue of the Rep domain was altered to alanine (two at a time), and mutants were assayed for the ability to repress a minimal AD2 domain. Two mutants, m4 and m6 (corresponding to residues 518–519 and 522–523, respectively) clearly affect the inhibitory function of Rep, which results in a dramatic restoration of AD2 transcriptional activity (Fig. 3C). Mutation of individual residues of the pairs had little effect by themselves (data not shown). These data suggest that residues Glu-518 and Glu-519, as well as Asn-522 and Leu-523 are crucial for Rep domain inhibitory function.

E12 Homodimers Bound to the MCK Enhancer Are Inactivated by the Rep Domain and AD1—Given that the Rep domain is highly conserved in E proteins (Figs. 1 and 3B), we utilized the E12 molecule exclusively for the remainder of the experiments because of its high expression level and transactivation potential in a variety of lymphocytic and myogenic genes. We sought to determine if the Rep domain was responsible for maintaining E protein homodimers in an inactive state on the MCK enhancer. As shown in Fig. 4A, MyoD strongly activates transcription from the MCK enhancer, whereas E12 is completely inactive. Deletion of the Rep domain alone (E12Δrep) had no effect on E12 activity. However, when activation domain 1 (AD1) was also deleted (E12Δrep ΔAD1), E12 became a potent transcriptional activator on the MCK enhancer. A molecule missing only AD1 (E12AD1), however, lacked detectable transactivation potential. These data suggested that both AD1 and Rep interfere with homodimer activation of the MCK enhancer. We also demonstrated that E12Δrep ΔAD1 drives the MCK enhancer via AD2, because deletion of AD2 (E12Δrep
FIG. 2. The Rep domain maps to positions 511–540. A, truncated versions of E2-2 were fused to the GAL4 DBD as indicated. GAL4-luciferase reporter plasmid was transfected into HeLa cells along with expression plasmid and assayed for luciferase activity after 48 h. B, Western blot of transfected cell nuclear protein. The lane labeled “mock” contains nuclear extract from mock transfected cells. Lanes 2–14 contain extracts from cells transfected with constructs described in rows 2–14 in A. C, electrophoretic mobility shift analysis of transfected cell nuclear protein. Transfected COS-7 cell nuclear extracts were used in an electrophoretic mobility shift assay using a radiolabeled GAL4 binding site probe. The lane labeled “mock” contains no added nuclear protein, and lanes 1–14 contain protein extracted from cells transfected with constructs described in rows 1–14 of A. The free probe is below the region of the gel displayed. D, truncated versions of E2-2 were cloned into pcDNAIII as diagrammed. 4RtkCAT [38] was transfected into HeLa cells along with the indicated expression plasmid and assayed for CAT activity after 48 h. Shown are scintillation counts per minute relative to amount of extract assayed. The values were corrected for the amount of total protein in the extracts. E, electrophoretic mobility shift analysis of transfected cell nuclear protein. Transfected COS-7 cell nuclear extracts were used in an electrophoretic shift assay using a radiolabeled E box probe. The lane labeled “mock” contains no added nuclear protein, and lanes 1–9 contain protein extracted from cells transfected with constructs described in rows 1–9 of D. Position of E2-2 protein homodimer-DNA complexes is indicated by E-E. NS refers to a nonspecific protein-DNA complex. The position of the free probe below the region of the gel displayed.
ΔAD1 ΔAD2) results in complete inactivation of the molecule. These results demonstrate that E proteins lacking a Rep do-

To evaluate this phenomenon on endogenous muscle gene expression, we performed immunofluorescence analysis on 10T1/2 cells transiently transfected with our E protein mu-

tants. 10T1/2 cells were cultured on chamber slides and trans-

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FIG. 3. The Rep domain inhibits both AD1 and AD2 but not VP16 and is conserved in all vertebrate E prote-

A, the E2-2 Rep domain (amino acids 502–540) was fused C-terminal to E2-2 AD1 (amino acids 1–225) and AD2 (amino acids 226–450), and to VP16 activ-

ation domain. These proteins were then fused to the GAL4 DBD. Indicated expression plasmids and GAL4-lucif-

erase reporter were cotransfected into HeLa cells and assayed for luciferase ac-

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rafts. rectangles and, and immunofluorescence was performed using the ClustalW alignment algorithm (42). Identical residues are indicated by dark shading, and similar amino acids are indicated by gray shading. C, Ala-

nine-scanning site-directed mutagenesis of the E2-2 Rep domain. Alanine substitu-

tions (two at a time) were made in E2-2 minimal Rep domain (residues 512–540). The wild type and mutated expression plasmids were cotransfected with GAL4-luciferase reporter plasmid into HeLa cells and assayed for lucifer-

ase activity after 48 h (as described under “Experimental Procedures”).
pothesized that activity of MyoD-E12 complexes is dependent on masking of the AD1 repressive activity by the Rep domain. MyoD-E12Δrep complexes would be unable to perform this masking event but should regain wild type activity if the relevant activation domain was inactivated. To test this, we made use of the E12 mutant proteins with targeted deletions of AD1 and AD2.

As shown in Fig. 5C, the E12 mutant lacking both Rep and AD1 domains (E12ΔRepΔAD1) are fully capable of acting synergistically with MyoD on the MCK enhancer, confirming our hypothesis. AD1’s repressive function in the MyoD-E12 complex was further supported by the observation that the E12 mutant lacking AD1 alone (E12ΔAD1) showed higher transactivation potential compared with wild type (Fig. 5C). In con-
Fig. 5. MyoD-E12 heterodimer activity requires inactivation of AD1 by the Rep domain. A, activity of E12Δrep complexed to MyoD on the MCK enhancer. Indicated amounts of E12 or E12Δrep plasmid were cotransfected with EMC11S and MCK luciferase into C3H10T1/2 cells. Cells were transferred to insulin transferrin medium (see “Experimental Procedures”) and assayed for luciferase activity 48 h later. B, electrophoretic mobility shift analysis of transfected cell nuclear protein. Transfected COS-7 cell nuclear extracts were used in an electrophoretic mobility shift assay using a radiolabeled E box site probe. The lane labeled “mock” contains no added nuclear protein. Lanes 1–3 contain extracts from cells transfected with the E12 constructs alone (pCDNA, E12, and E12Δrep, respectively), whereas lanes 4–6 contain the extracts with MyoD cotransfected with them individually. Position of E12 protein homodimer-DNA and E12-MyoD heterodimeric complexes is indicated by E-E and MyoD-E, respectively. C, synergistic activity of E12 deletion mutants with MyoD on the MCK enhancer. 0.5 mg of E12 and 0.5 mg of MCK luciferase were cotransfected into C3H10T1/2 cells with or without 0.1 mg of EMC11S. Cells were transferred to insulin transferrin medium (see “Experimental Procedures”) and assayed for luciferase activity 48 h later. Synergy was calculated by dividing the activity of cotransfected samples by the activity of individually transfected samples. All transfections were performed at least three times in triplicate.
trast, mutants with targeted deletions of AD2 are inactive in MyoD-dependent transcription, both in the presence and absence of the Rep domain. Electrophoretic mobility shift analysis using extracts from transfected cells demonstrates that these activity differences are not the result of altered expression or DNA binding efficiency (data not shown). These results demonstrate that the MyoD-E heterodimer stimulates a myogenic enhancer via AD2 and requires Rep-domain-mediated repression of AD1 (see model, Fig. 6B). Thus the AD1 domain of E protein is repressive on the MCK enhancer, both in the context of E protein homodimer or MyoD-E protein heterodimer, and its repression activity is suppressed by the Rep domain only as a heterodimer (see model, Fig. 6).

DISCUSSION

E protein complexes are potent transcriptional activators and are highly specific for the enhancers they stimulate. As homodimers, E proteins directly stimulate transcription from the B lymphocyte-specific IgH and Igk enhancers and thereby facilitate immunoglobulin gene rearrangement (14, 15, 34–37). E protein homodimers also directly or indirectly stimulate the terminal deoxynucleotidyl transferase (TdT) I, EBF, and RAG genes in vivo (18, 38). As heterodimers with MyoD, E protein complexes stimulate transcription of muscle-specific genes such as muscle creatine kinase (MCK) and myosin light chain (39). MyoD-E protein heterodimers bind E boxes in the immunoglobulin enhancer but are transcriptionally inactive as a result of a cis-acting repression region on the enhancer (28).

Conversely, E protein homodimers bind E boxes in the MCK enhancer, but their activities are completely repressed. The mechanisms the cell uses for recognition and inactivation of E protein homodimers bound to non-lymphocyte enhancers are largely unknown.

In this study, we have identified a novel cis-acting transcriptional repression domain in the E proteins that plays a role in modulating the activity of bHLH complexes in an enhancer-specific manner. The Rep domain is a charged 30-amino acid region, highly conserved among the vertebrate E proteins, which is capable of repressing E protein activation domains 1 and 2. In E protein homodimers bound inappropriately to a myogenic enhancer, the Rep domain and AD1 cooperate in preventing transcriptional activation. In MyoD-E protein heterodimers bound to the MCK enhancer, the Rep domain is critical for transcriptional activation by masking negative signals derived from AD1. The Rep domain, then, is an intramolecular transcriptional repressor that plays a role in modulating the activity of enhancer-bound bHLH protein complexes.

The Rep domain is a potent transcriptional repressor that inhibits the activities of the AD1 and AD2 domains of the E proteins. This repression is somewhat specific to these domains, because VP16 activity is unaffected by the addition of this domain. Work to determine Rep domain-interacting partners is ongoing.

We have demonstrated that AD1 is repressive for the MCK enhancer both in the context of E protein homodimeric and MyoD-E heterodimeric complexes. This domain is normally active in E protein homodimers and is indeed required for activation of the immunoglobulin enhancer (data not shown). In MyoD-E heterodimers, AD1 is masked by the Rep domain, which allows AD2 to stimulate myogenic enhancers. The mechanism by which AD1 represses myogenic enhancers is unknown. We propose that either AD1 recruits a transcriptional repressor when bound to the MCK enhancer or AD1-repressed transcriptional coactivators interfere with myogenic enhancer function. These possibilities are currently being evaluated.

The Rep domain may inhibit AD1’s repression function in E protein heterodimers by preventing the recruitment of RNAPII or coactivators, such as histone acetyltransferase containing transcriptional cofactor, SWI/SNF complexes or mediators (40, 41). We suspect, therefore, that the Rep domain is capable of preventing the assembly of several different transcriptional cofactor complexes. The simplest model we proposed in Fig. 6 with multiple functional interactions observed between AD1, AD2, and Rep domain is that multiple, sequential intramolecular interactions contribute to transactivation specificity. We presume that this kind of complex intramolecular regulation in E proteins exists in part to permit a broad range of regulatory capabilities.

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FIG. 6. Intramolecular regulations in E protein activation domain utilization. A, when E protein homodimers are bound to the MCK enhancer, AD2 is repressed by the Rep domain and AD1 interferes with enhancer function. B, when MyoD-E heterodimers are bound to the MCK enhancer, AD2 is active and drives transcription (possibly in cooperation with activation domains in MyoD) and AD1 is repressed by the Rep domain.
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