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DEC1 Negatively Regulates the Expression of DEC2 through Binding to the E-box in the Proximal Promoter

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Human DEC (differentially expressed in chondrocytes), mouse STRA (stimulated with retinoic acid), and rat SHARP (split and hairy related protein) proteins constitute a new and structurally distinct class of the basic helix-loop-helix proteins. In each species, two members are identified with a sequence identity of >90% in the basic helix-loop-helix region and ~40% in the total proteins, respectively. Recently, we have reported that DEC1 is abundantly expressed in colon carcinomas but not in the adjacent normal tissues. The present study was undertaken to extend the expression study of DEC1 and to determine whether DEC1 and DEC2 had similar expression patterns among paired cancer-normal tissues from the colon, lung, and kidney. Without exceptions, DEC1 was markedly higher in the carcinomas, whereas the opposite was true with DEC2. In stable transfectants, tetracycline-induced expression of DEC1 caused proportioned decreases in the expression of DEC2. Co-transfection with DEC1 repressed the activity of a DEC2 promoter reporter by as much as 90%. The repression was observed with wild type DEC1 but not its DNA binding-defective mutants. Studies with deletion and site-directed mutants located in the proximal promoter, an E-box motif that supported the DEC1-mediated repression. Disruption of this E-box markedly abolished the ability of the reporter to respond to DEC1. Our findings assign for DEC1 the first target gene that is regulated through direct DNA binding. DEC/STRA/SHARP proteins are highly identical in the DNA binding domain but much more diverse in other areas. DEC1-mediated repression on the expression of DEC2 provides an important mechanism that these transcription factors regulate the cellular function not only by modulating the expression of their target genes but also the expression of members within the same class.

The basic helix-loop-helix (bHLH) proteins are intimately associated with developmental events such as cell differentiation and lineage commitment (1–6). The HLH domain in the bHLH motif is responsible for dimerization, whereas the basic region mediates DNA binding (1). Based on sequence alignment and domain analysis, human DEC (differentially expressed in chondrocytes), mouse STRA (stimulated with retinoic acid), and rat SHARP (split and hairy related protein) constitute a new and structurally distinct class of bHLH proteins (7–10). These proteins are distinctly related to Drosophila Hairy and E(spl) as well as the mammalian homologues (e.g. HES) with the highest sequence identity (~40%) in the bHLH region (1, 11, 12). Like Hairy/E(spl)/Hes, DEC/STRA/SHARPs contain an orange domain and a proline residue in the DNA binding domain. However, the proline is located 2 residues more toward the NH2-terminus (1, 8). Another major structural difference on the functional domains is that DEC/STRA/SHARPs, unlike Hairy/E(spl)/Hes proteins, lack the COOH-terminal WRPW tetrapeptide motif (13). Through this sequence, Hairy/E(spl)/Hes recruit corepressor Groucho to the transcription regulatory complex (13). Recruitment of Groucho is responsible for a vast array of biological activities of Hairy/E(spl)/Hes proteins including cell differentiation and lineage commitment (14–18).

Two members of DEC/STRA/SHARP proteins are identified in each mammalian species studied with a sequence identity of >90% in the bHLH region and ~40% in the total proteins, respectively (8). They exhibit an overlapping tissue distribution, and their expression is highly elevated in response to environmental stimuli (7–10). In rats that undergo seizure induction by kainic acid, the levels of mRNA encoding SHARP1 or 2 are sharply increased within 1 h in the brain (9). In cultured human cells, both DEC1 and DEC2 are markedly induced in response to hypoxia (19). Co-transfection experiments with promoter reporters have identified functional hypoxia response elements in both DEC1 and DEC2 genes. These elements show high affinity toward hypoxia-inducible factor-1α and -β, providing a molecular explanation on the co-regulatory phenomena of DEC1 and DEC2 during hypoxia response (19). Rapid induction of these proteins in response to environmental stimuli suggests that DEC/STRA/SHARPs are protective against detrimental conditions.

In addition to a potential protective role against environmental stimuli, DEC/STRA/SHARPs have been implicated in cell differentiation (7, 10, 20), maturation of lymphocytes (21), and regulation of molecular clock (22). In a cell culture system, mouse STRA13 promotes neuronal but represses mesodermal and endodermal differentiation (7). Consistent with the inductive effect on neuronal differentiation, rat SHARP proteins are abundantly expressed in a subset of mature neurons (9). DEC1 has recently been shown to promote chondrocyte differentiation at the early and terminal stages (20). STRA13-deficient mice, although surviving to adulthood, develop autoimmune diseases accompanied by accumulation of spontaneously activated T and B cells (21). In addition, the mouse proteins are recently found to regulate the expression of biological clock regulator Per (22). Recently, we and other investigators have
recently demonstrated that deregulated cell survival by DEC1 may have oncogenic significance. In paired samples, DEC1 is abundantly expressed in colon carcinomas but not in the adjacent normal tissues (23). High levels of DEC1 transcript are also detected in an array of cancer cell lines derived from a wide range of organs (24). Cells that lack the functional tumor suppressor VHL (von Hippel-Lindau) express higher levels of DEC1 (24). Forced expression of DEC1 antagonizes serum deprivation-induced apoptosis and selectively inhibits the activation of proapoptotic (23). These findings suggest that overexpression of DEC1 provides cells with an unusual survival mechanism and thus is oncogenic.

The present study was undertaken to extend the expression study on DEC1 and to determine whether DEC1 and DEC2 displayed similar expression patterns among paired tumor-normal tissues from the colon, lung, and kidney. Without exceptions, DEC1 was expressed markedly higher in the carcinomas, whereas DEC2 was expressed markedly higher in the adjacent normal tissues. Forced expression of DEC1 sharply decreased the expression of DEC2 and markedly repressed the activity of a DEC2 promoter reporter. Co-transfection experiments with mutant reporters and electrophoretic mobility shift assay (EMSA) located, in the proximal promoter, an E-box that supports DEC1-mediated repression. These findings provide direct evidence that DEC1 negatively regulates the expression of DEC2 and provides a mechanism that could be used to explain why DEC1 is expressed at such high levels in colon carcinomas, whereas DEC2 is expressed at much lower levels.

**SITE-DIRECTED MUTAGENESIS**—The DEC2 promoter reporter had two E-box motifs that probably interact with DEC1, and the studies with deletion mutants suggested that the E-box in the proximal region supports DEC1-mediated repression. In order to definitively establish such a role, site-directed mutagenesis was performed to substitute two of the six nucleotides. The mutant construct was prepared with a QuikChange site-directed mutagenesis kit (Stratagene). Complementary oligonucleotides (5'-GATGGTACGTTCCGAGCGGAGTGGTTGCTGG-3') were synthesized to target this region. To perform the substitutions, the primers were annealed to a DEC2 promoter reporter amplified to a thermostable product for a total of 15 cycles. The PCR-amplified constructs were then digested with DpnI to remove the nonmutated parent construct. The mutated PCR-amplified constructs were used to transform XL1-Blue. The same approach was used to prepare three DEC1 mutants that had single or double residues substituted in the DNA binding domain (P56A, R58P, or both). The general sequence for the site-directed mutagenic oligonucleotides was 5'-GAGACCTAACATGGGCGGACCGCTGATCGGAGAAAAAG-3' with the nucleotides in boldface type substituted individually or simultaneously. All mutated constructs were subjected to sequencing analysis to confirm the desired mutation being made without secondary mutations.

**CO-TRANSFECTION EXPERIMENT**—Cells (293T) were plated in 24-well plates in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at a density of 1.6 × 10^5 cells/well. Transfection was conducted by lipofection with LipofectAMINE according to the manufacturer’s instructions. Transfection mixtures contained DEC1 or a mutant construct (100 ng), reporter plasmid (100 ng), and the pRL-TK Renilla plasmid (1 ng). If a DEC1-stable line was used, DEC1 or its mutant construct was omitted from the transfection mixture. The transfected cells were cultured for an additional 24 h, washed once with phosphate-buffered saline, and resuspended in passive lysis buffer (Promega). The lysed cells were subjected to two cycles of freezing/thawing. The reporter enzyme activities were assayed with a Dual-Luciferase Reporter Assay System. The dual luciferase system was used to determine the activity of the two luciferases sequentially. The first luciferase activity, which represented the reporter gene activity, was initiated by mixing an aliquot of lysates (20 μl) with Luciferase Assay Reagent II. Then the second luciferase activity was measured. The Renilla activity was simultaneously activated by adding Stop & Glo reagent to the sample wells. The firefly luminescence signal was normalized based on the Renilla luminescence signal. In cases where the reading on the luciferase activity was too high, the lysates were diluted, and luciferase activities were then determined to minimize the interference on the reading of the Renilla luciferase activity.

**EMSA**—Cells (293T) were transfected with DEC1 or a mutant, and nuclear extracts were prepared with a nuclear extract kit (Active Motif). In some cases, DEC1-stable transfected cells were used but cultured in the presence or absence of tetracycline to modulate the expression of transfected DEC1. Nuclear proteins (10 μg) were incubated with radiolabeled double-stranded oligonucleotides (5'-CGTTTACGATCGAGCGGAGTGGTTGCTGG-3') in a final volume of 10 μl containing 1× DNA binding buffer. For supershift experiments nuclear extracts were first incubated with a 10- or 50-fold molar excess of cold probe and then mixed with the radiolabeled probe. Oligonucleotides with a disrupted E-box were also used in the competition assays. For supershift assays, the anti-DEC1 or an anti-FLAG antibody was added either before or after the nuclear extracts were incubated with the radiolabeled probe. The protein-DNA complexes were resolved in 6% PAGE and visualized by autoradiography.

**OTHER ANALYSES**—Western analyses were conducted as described previously (27). The anti-DEC1 antibody against the COOH-terminal peptide was described elsewhere (23). Protein concentration was determined by the Bradford method.
RESULTS

DEC1 and DEC2 Are Inversely Expressed in Paired Carcinomas and Adjacent Normal Tissues—We have reported that DEC1 is abundantly expressed in colon carcinoma but not in the adjacent normal tissues (23). The initial focus of the present study was to extend the expression study on DEC1 and to determine whether DEC1 and DEC2 shared similar expression patterns among paired cancer-normal tissues from the colon, kidney, and lung. RT-PCR experiments with primers specific to DEC1 and DEC2 were performed. As shown in Fig. 1, without exceptions, the levels of DEC1 mRNA were markedly higher in the carcinomas, whereas the levels of DEC2 mRNA were markedly higher in the adjacent normal tissues. Between paired samples, the levels of β-actin mRNA were comparable. The carcinoma-related increase in DEC1 expression was also detected by Western blot (top of each depicted figure), suggesting that mRNA levels are indicative of the overall expression of these two genes.

Forced Expression of DEC1 Proportionally Decreases the Expression of DEC2—The inversed expression patterns between DEC1 and DEC2 suggest that DEC1 negatively regulates the expression of DEC2 or vice versa. In order to directly test this possibility, DEC1-stable transfected lines were used to study the expression relationship between DEC1 and DEC2. Two clonal stable lines were included: one expressing DEC1 (wild type) and the other expressing DEC1-M, which lacked the DNA binding domain. The stable lines were prepared with 293T cells and the pcDNA6/TR-pcDNA4 expression system; therefore, the expression of DEC1 and DEC1-M was inducibly regulated by tetracycline as described previously (23). As expected, the addition of tetracycline caused a concentration-dependent increase on the levels of DEC1 as determined by Western blots (Fig. 2A, top). Consistent with the inducible increase in the levels of DEC1 protein, the levels of DEC1 mRNA were proportionally increased (data not shown). In contrast to the increased expression of DEC1, the levels of DEC2 mRNA were proportionally decreased (Fig. 2A). However, such inversely expressed patterns were observed only in the cells expressing wild-type DEC1 (Fig. 2A) and not the cells expressing the DEC1 mutant, although the levels of DEC1-M were markedly induced by tetracycline (Fig. 2B).

The E-box in the Proximal Promoter of DEC2 Is the Sequence Targeted by DEC1—The inability of DEC1-M to down-regulate the expression of DEC2 suggests that DEC1-mediated repression is achieved through a DNA-binding mechanism. In order to directly test this possibility, reporter experiments and EMSA were conducted. A DEC2 promoter reporter (pLuc-1888) was constructed to contain the basal promoter and other potential regulatory sequences of the DEC2 gene (−1,888 to +11). This region was chosen because it contained two E-box motifs that commonly serve as target sequences for bHLH transcription factors (1). A series of 5′ deletion mutants of this reporter was also prepared and designed to specify the location of DNA sequence that is targeted by DEC1 (Fig. 3A, left). Co-transfection experiments were conducted to test these reporters for their ability to support DEC1-mediated activity. The stable transfected line (wild-type DEC1 only) was transfected again with a reporter construct and cultured in the presence or absence of tetracycline to modulate the expression of DEC1. The pRL-TK Renilla plasmid was also included in the transfection mixture to normalize transfection efficiency. As described in Fig. 3A (right), the addition of tetracycline decreased the activity of the pLuc-1888 reporter by as much as 90%. Similar repression was observed with the reporters that had the sequence deleted up to nucleotide −535. In contrast, reporter pLuc-125, which had a further deletion from nucleotide −535 to −125, simultaneously lost the basal transcription activity and the ability to respond to DEC1, suggesting the importance of this region (−535 to −125) in both basal and regulatory transcription.

We next examined whether responsiveness to DEC1 could be separated from the basal transcription activity in the DEC2 promoter reporter. Given the fact that this region (−535 to −125) contains a single E-box that is probably targeted by
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Double-stranded oligonucleotides harboring this E-box were synthesized and radiolabeled. The labeled probe was incubated with the nuclear extracts and analyzed by EMSA. As shown in Fig. 3B, incubation with the extracts from the cells cultured in the presence of tetracycline yielded a shifted band (lane 8). This band was not detected when incubation was performed with the extracts from the cell cultured without tetracycline (lane 7). The shifted band was competed completely by 50× (lane 1) or partially by 10× excess cold probe (lane 3). However, the oligonucleotides (50×) that harbored a mutated E-box (E-box-M) showed no competitive activity (lane 2). In addition, the shifted band was supershifted by the anti-DEC1 but not the anti-FLAG antibody. The supershifted band appeared whether the antibody was added before or after the DEC1-DNA complexes were formed (lanes 5 and 6), suggesting that the antibody binding does not interfere with interactions between DEC1 and its element (the antibody directed against the COOH-terminal sequence of DEC1).

DNA Binding Is Required to Effectively Repress the DEC2 Promoter Reporter—Disruption of the proximal E-box caused drastic but incomplete loss of responsiveness to DEC1 (Fig. 3A), suggesting that DNA binding is not the only mechanism involved in DEC1-mediated repression on the DEC2 reporter or that an additional DEC1 binding site exists in this region. We next tested whether DEC1 mutants, defective of DNA binding, had any repressive activity. These mutants had one or more residues in the DNA binding domain substituted or one or more structural domains deleted (Fig. 4A). A total of three deletion mutants (DEC1-M, DEC1105–412 and DEC1327–412) were prepared, and all of them lacked the DNA binding domain. As shown in Fig. 4A, additional sequences were also deleted in DEC1105–412 (the HLH motif) and DEC1327–412 (the HLH motif and orange domain). The HLH motif and the orange domain are shown in other bHLH proteins to mediate dimerization and protein interactions, respectively (1). Similarly, three substitution mutants were prepared, including DEC1P56A, DEC1R58P, and DEC1P56A/R58P. The rationale for preparing the substitution mutants was that proline 56 was assumed to be critical in DNA binding based on studies with other bHLH proteins (1). However, there is a major difference regarding the location of this proline. In other bHLH proteins, the proline is located 2 residues more carboxyl terminal (corresponding to residue 58 in DEC1) (7, 8). Therefore, the mutants represented substitution of proline 56 with an alanine (DEC1P56A), arginine 58 with an alanine (DEC1R58P), or both (DEC1P56A/R58P).

Co-transfection experiments were conducted to test these DEC1 mutants for their ability to repress the DEC2 reporter (pLuc-1888). As shown in Fig. 4A (top), all deletion mutants (DEC1-M, DEC1105–412, and DEC1327–412) exhibited little repressive activity toward this reporter. In contrast, all substitution mutants repressed the DEC2 reporter, but the overall repressive activity varied markedly among them. The DEC1P56A mutant showed a similar potency as the wild-type DEC1 (~90% repression), whereas the other two mutants (DEC1R58P and DEC1P56A/R58P) caused only ~65% and ~50% repression, respectively. The expression of DEC1 and its mutants was comparable with the exception of DEC1-M that was expressed to a higher extent (Fig. 4B), excluding the possibility that lack of expression was a contributing factor to the weaker repression by some of the mutants (e.g. DEC1P56A/R58P). In order to determine whether these mutants, particularly the mutants DEC1R58P and DEC1P56A/R58P, indeed lost DNA binding ability, nuclear extracts from the respective transfected cells were incubated with the radiolabeled E-box oligonucleotides, and the corresponding DNA-protein complexes were analyzed by EMSA. As predicted, all deletion mutants (DEC1-M,
DEC1-mediated repression on the DEC2 promoter reporter and binding to the proximal E-box. A. DEC1-mediated repression on the DEC2 promoter reporter. Deletion and site-directed mutants of the DEC2-promoter reporter (pLuc-1888) were prepared by endonuclease digestion followed by ligation or by PCR with a QuickChange site-directed mutagenesis kit. DEC1-stable transfected cells were cultured in 24-well plates at ~80% confluence and transfected again with a reporter construct (100 ng) and the pRL-TK Renilla (1 ng). The retransfected cells were cultured in the presence or absence of tetracycline (1 μg/ml) for 24 h. The cells were collected, washed once with phosphate-buffered saline, and resuspended in passive lysis buffer. The reporter enzyme activities were assayed with a Dual-Luciferase reporter assay system. The firefly luminescence signal was normalized based on the Renilla luminescence signal. B. EMSA-DEC1-stable transfected cells were cultured in the presence or absence of tetracycline (tet; 1 μg/ml) for 24 h, and nuclear extracts were prepared with a nuclear extraction kit (Active Motif). Nuclear proteins (10 μg) were incubated with radiolabeled double-stranded oligonucleotides harboring the proximal E-box in a final volume of 10 μl containing 1× DNA binding buffer. For competition experiments, nuclear extracts were first incubated with excess cold probe (50 μM in lane 1 or 10 μM in lane 3) and then mixed with the radiolabeled probe. Oligonucleotides (M) with the E-box disrupted were also used in the competition assays (50 μM in lane 2). For supershift assays, the anti-DEC1 antibody (D) was added either before (lane 5) or after (lane 6) the nuclear extracts were incubated with the radiolabeled probe. As a control, the anti-DEC1 antibody was replaced by an anti-FLAG antibody (F, lane 4). The protein-DNA complexes were resolved in 6% polyacrylamide gel electrophoresis and visualized by autoradiography.

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Dec1_105-412, and Dec1_237-412) showed no DNA binding activity (result shown for Dec1-M only) (Fig. 4C). In contrast, DNA binding activity varied among the substitution mutants. Dec1_H56A and Dec1_R56R, had no DNA binding activity, consistent with the fact that Dec1_H56A was the only substitution mutant that effectively repressed the promoter activity of DEC2 (Fig. 4A).

DNA Binding Is Not Sufficient to Confer Repressive Activity—The studies with DNA binding defective mutants clearly demonstrated the importance of DNA binding in repressing the DEC2 promoter. We next examined whether DNA binding was sufficient to exert repression. In order to directly test this possibility, DEC1 mutants were prepared to keep the bHLH motif intact (DNA binding) but have sequences with various lengths deleted from the COOH terminus (Fig. 5A). These COOH-terminal truncated mutants were subcloned in the FLAG vector to facilitate immunodetection. Similarly, co-transfection experiments were performed with DEC1 or a mutant along with the DEC2 reporter (pLuc-1888). As shown in Fig. 5A, deletion of the COOH-terminal 65 residues (FLAG–DEC1_1–150) caused no changes in the repressive activity (1). In contrast, deletions of additional COOH-terminal sequence caused a partial or a complete loss of repressive ability. As a matter of fact, FLAG–DEC1_1–150 no longer had any repressive activity.

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Next, we examined whether these mutants actually retained DNA binding activity. EMSA was performed with the nuclear extracts from the cells used for reporter assays. As shown in Fig. 5C, a shifted band was detected with all COOH-terminal
truncated mutants. The relative electrophoretic mobility was generally associated with the size of a mutant. For example, FLAG-DEC11–150 was the shortest among the mutants, and the shifted band with this mutant exhibited the fastest mobility. More importantly, the addition of an anti-FLAG antibody into the binding reactions resulted in the appearance of a super-shifted band accompanied by the disappearance of the original shifted band, providing direct evidence that the observed protein-DNA interactions were highly specific. These findings also suggest that DNA binding, although essential, is not sufficient to confer repressive effect.

The HLH Motif Is Required for Dominant Interfering Regulation—The inability of FLAG-DEC11–150 to exert repression, although it bound effectively to DNA, points to two important possibilities: the deleted region from residue 150 to 347 has intrinsic repressive activity, or this region is responsible for recruiting protein(s) that causes repression. Apparently, comprehensive experiments are required to definitively establish the involvement of each possibility. However, we examined the second possibility by testing mutants that contained part or the entire sequence of this region for the ability to function as a dominant interfering regulator. Co-transfection experiments were conducted with DEC1 in the presence and absence of a mutant. Among mutants DEC1-M, DEC1105–412, and DEC1237–412, only DEC1-M effectively reversed DEC1-mediated repression (Fig. 6A), although they all shared two important features; they lacked the entire DNA binding domain and lacked repressive activity themselves (Fig. 4A). Among the substitution mutants, DEC1R58P and DEC1P56A/R58P but not DEC1P58A partially but significantly reversed DEC1-mediated repression, consistent with the fact that DEC1P58A was a potent repressor itself (as potent as wild type DEC1), whereas DEC1R58P and DEC1P56A/R58P were much less repressive (Fig. 4A). It should be emphasized that the expression patterns in the cells co-transfected with DEC1 and a mutant were consistent with what was predicted; a band with more intensified staining was detected if a mutant co-migrated with DEC1 (e.g. DEC1P56A/R58P); otherwise, an additional band (e.g. DEC1105–412) was detected if a mutant and DEC1 were electrophoretically distinct.

We also tested all COOH-terminal truncated mutants for the ability to function as dominant interfering regulators. Generally, these mutants either partially or completely antagonized DEC1-mediated repression, depending on the relative potency to act as a repressor by its own (Fig. 6B). Mutants with less intrinsic repressive activity exhibited a higher potency to reverse the repression by DEC1. For example, FLAG-DEC11–150 itself had no repressive activity (Fig. 4A) but completely reversed DEC1-mediated repression (Fig. 6B). Among all mutants that were less repressive than wild type DEC1, only DEC1105–412 and DEC1237–412 failed to reverse the repression by DEC1 (Fig. 6), and they were the only mutants that did not contain the HLH domain (Figs. 4A and 5A), suggesting that the dominant interfering regulatory activity is achieved through the HLH domain. The HLH domain is known to mediate dimerization (1), and mutants with an intact HLH domain probably
form dimers with wild type DEC1, but the resultant dimers
have no DNA binding activity or are transcriptionally inactive.
In support of the first possibility, we performed EMSA and found that DEC1R58P and DEC1P56A/R58P (DNA binding-defec-
tive mutants) markedly abolished the DNA binding ability of
DEC1 when cells were co-transfected together with DEC1 and
DEC1R58P or DEC1P56A/R58P (data not shown).

DISCUSSION

The bHLH proteins are intimately associated with develop-
mental events such as cell differentiation and lineage commit-
tment (1). Based on sequence alignment and functional domain
analyses, human DEC proteins, along with mouse STRA and
rat SHARP, constitute a new class of bHLH transcription fac-
tors (7–10). These proteins are shown to play important roles in
cell differentiation, regulation of molecular clock, immune re-
response, and xenobiotic response (7, 10, 19–22, 28). Recently,
we have reported that DEC1 is abundantly expressed in colon
carcinomas, antagonizes serum deprivation-induced apoptosis,
and selectively inhibits the activation of procaspases (23). In this
report, we describe inversed expression patterns between DEC1
and DEC2 among paired tumor-normal samples from the colon,
lung, and kidney. Experimentally forced induction of DEC1
causes proportional decreases in the expression of DEC2. Given
the fact that DEC/STRA/SHARP proteins are highly identical
(40%) in the DNA binding region, but very diverse in other
areas (<40%), our findings described in this report provide an
important mechanism by which the cellular function of target
genes probably shared by these proteins can be coordinately
affected by members within the same class.

DNA binding is probably the primary mechanism responsi-
bile for DEC1-mediated repression on the expression of DEC2,
although members of DEC/STRA/SHARP protein family have
been shown to use non-DNA binding mechanism(s) (29–31).
Several lines of evidence presented in this study support this
notion. First, studies with deletion and site-directed reporter
mutants identify the proximal E-box that supports the repres-
sion by DEC1. This E-box exhibits a high affinity toward DEC1,
and disruption of this E-box markedly reduces its responsive-
ness to DEC1 (Fig. 3), suggesting that DNA binding is involved
in the DEC1-mediated repression. Second, DEC1 deletion mu-

Fig. 5. DNA binding is not sufficient for DEC1 to repress DEC2 pro-

FIG.5 .

DNA binding is not sufficient for DEC1 to repress DEC2 pro-
moter activity. A, co-transfection exper-
iment. Cells (293T) were cultured in 24-
well plates and transiently transfected
with DEC1 or a COOH-terminal trun-
cated mutant (100 ng), DEC2 promoter
reporter (pLuc-1888; 100 ng), and the
pRL-TK Renilla (1 ng). Determination
and calculation of the luciferase activities
were described in the legend to Fig.
1. C, EMSA. Nuclear contracts were pre-
pared from cells transiently transfected
with DEC1 or a mutant and incubated
with radiolabeled oligonucleotides har-
boring the proximal E-box. Similarly,
competition experiments were performed
with excess cold probe (E) or a mutant
probe (M) as described in the legend to
Fig. 3B. For supershift assays, an anti-
FLAG (F) or the anti-DEC1 (D) was added
to the incubation mixtures before being
analyzed by PAGE.
sion, is not sufficient to repress the DEC2 promoter. Mutant FLAG-DEC1\textsubscript{1-150}, for example, binds effectively to DNA but shows no repressive activity (Fig. 5A). As a matter of fact, mutants, with a deletion in the region from residue 150 to 347, all bind to DNA as effectively as wild type DEC1 but are markedly less repressive (Fig. 5A). In this region, several helical structures and particularly an orange domain are located (7, 8). These structures are assumed to mediate protein-protein interactions based on studies with other bHLH proteins (1, 7, 8). It is likely that this region recruits proteins that cause repression. However, the necessity of protein recruitment to repress DEC2 is unlikely, because mutants such as DEC1\textsubscript{105-412} contain the entire sequence of this region but show no dominant interfering activity against wild type DEC1 (Fig. 6A). In this study, we have demonstrated that the contribution of Pro\textsubscript{56} to DNA binding is insignificant because mutant DEC1 P58A is equally effective as wild type DEC1 in DNA binding (Fig. 4C). In contrast, introduction of a proline by substituting Arg\textsubscript{58} completely eliminates DNA binding activity (Fig. 4C), suggesting that residue in this location is indeed important for E-box binding. It would be interesting to test whether DEC1R58P and DEC1 P56A/R58P show an increase in binding to an N-box sequence. In addition, the DEC2 reporter contains two identical E-box sequences (proximal and distal) (Fig. 3A); however, only the proximal E-box is required for responding to the repression by DEC1. The precise mechanism for such a difference remains to be determined. It is likely that the genomic context rather than an E-box alone determines

FIG. 6. Dominant interfering regulation on DEC1-mediated repression. A, effects of DNA binding-defective mutants on the repressive activity by DEC1. Cells (293T) were cultured in 24-well plates and transiently transfected with DEC1 (50 ng) in the presence or absence of a DNA binding-defective mutant (100 ng). Vector construct was used to equalize the amount of plasmid in each transfection. Similarly, the pRL-TK Renilla plasmid (1 ng) was included in the transfection mixture to normalize transfection efficiency. Determination and calculation of the luciferase activities were described in the legend to Fig. 4. To determine the expression levels of transfected constructs, cell lysates (10 \mu g) from the cells used for reporter activity were analyzed for the expression of DEC1 and its mutants by the anti-DEC1 antibody (specific to the COOH terminus of DEC1). B, effects of the COOH-terminal truncated mutants on the repressive activity by DEC1. The co-transfection and immunodetection were performed as described in the legend to Fig. 6A. However, both anti-DEC1 and anti-FLAG antibodies were simultaneously used for the immunodetection, because the COOH-terminal truncated constructs were prepared with the FLAG-cytomegalovirus vector.
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intracellular DNA binding. In support of this possibility, STRA13 has been shown to preferentially bind to an E-box flanked with certain nucleotides.

DECa and DECb share the DNA binding domain with an exception of a single residue (aspartate versus glutamate, the farthest NH2-terminal residue of this domain) (8); therefore, DECb probably acts as an autoregulator. This possibility is further supported by their highly identical sequences flanking the DNA binding domain. Immediately COOH-terminal to the DNA binding domain is the helix-loop-helix domain that is identical between DECa and DECb, and NH2-terminal to this domain is an acidic residue-rich stretch in both proteins (8). The DNA binding domain and its highly identical flanking sequences suggest that DECa and DECb have overlapping target genes, particularly those that are regulated through direct DNA binding. In support of this notion, mouse proteins (STRA13 and DECb) have been recently shown to repress Clock/Bmal1-induced activation of the Per promoter (22), a gene that is involved in the regulation of the molecular clock. Therefore, it is likely that DECa/STRA/SHARP proteins are functionally redundant on some target genes, and such a redundant mechanism provides a possible explanation that STRA13 knockout mice develop to adulthood and show no discernible phenotypic differences compared with their wild-type littermates (21). It should be emphasized, however, that DECa and DECb may not necessarily exert the same biological activity on all target genes and in all cell types, particularly given the fact that they have a minimal sequence identity (<40%) in the COOH-terminal half and exhibit several major structural differences (8). Both DECa and DECb have an orange domain (two helical structures spanned by ~50 residues); however, the overall sequence identity in this domain is only moderate (~50%). In addition, an alanine/glycine-rich region is present in DECb but absent in DECa. Previous studies with STRA13 as well as the findings described in this study have demonstrated that the region harboring the orange domain is required to exert effective repression by both proteins (Fig. 5A) (7). Amino acid repeats, on the other hand, are implicated in protein folding, protein-protein interactions, and degradation (34).

DECa-mediated repression is probably responsible for the differences on cell and tissue distributions between DECa and DECb. Although Northern analyses have shown that DECa and DECb have an overlapping tissue distribution (8, 10), it remains to be determined whether they are actually expressed in the same cell type and to a similar extent (8, 10). Some organs with high levels of DECa (e.g. liver) express lower levels of DECb (10). Very recently, DECa and DECb were found to regulate the mammalian molecular clock, but they exhibit distinct and area-dependent expression patterns in the brain (21).

In this report, we have demonstrated that these two proteins exhibit inverted expression patterns among paired samples from the colon, kidney, and lung. An inducible expression system demonstrates that increased expression of DECa proportionally decreases the expression of DECb. The DECa-mediated repression is primarily achieved by binding to the E-box in the proximal promoter of DECb. Site-directed mutagenesis studies show that arginine 55 in the DNA binding domain is critical for DECa to interact with this E-box. Given the fact that DECa/STRA/SHARP proteins are emerging as very important regulators in a vast array of cellular events including cell differentiation, maturation of lymphocytes, oncogenesis, molecular clock, and xenobiotic response, findings described in this study provide an important mechanism by which these proteins regulate the cellular function by not only modulating the expression of their target genes but also the expression of the members within the same class.

REFERENCES


DEC1 Negatively Regulates the Expression of DEC2 through Binding to the E-box in the Proximal Promoter
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