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**THE DEC1 TRANSCRIPTION FACTOR:
ONCOGENIC INVOLVEMENT AND MOLECULAR MECHANISMS ON
TRANSCRIPTION REGULATION**

BY

YUXIN LI

**A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
PHARMACOLOGY AND TOXICOLOGY**

UNIVERSITY OF RHODE ISLAND

2003

DOCTOR OF PHILOSOPHY DISSERTATION

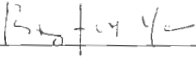


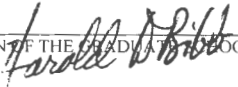
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YUXIN LI

APPROVED:

Dissertation Committee:

Major Professor

DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND
2003

ABSTRACT

The basic helix-loop-helix (bHLH) proteins are intimately associated with developmental events such as cell differentiation and lineage commitment. The HLH domain in the bHLH motif is responsible for dimerization whereas the basic region mediates sequence specific DNA binding. Human DEC, mouse STRA and rat SHARP proteins represent a new class of bHLH proteins. In each species, two members are identified with a sequence identity of >90% in the bHLH region and ~40% in the total protein, respectively. Based on sequence alignment and domain analysis, DEC/STRA/SHARP proteins show high similarities (~40%) to *Drosophila* Hairy and E(spl) as well as the mammalian homologues (e.g., HES) in the bHLH domain. However, they lack the C-terminal WRPW motif which is present at all other Hairy/E(spl)/Hes proteins and mediates transcription repression. These structural differences distinguish DEC/STRA/SHARPs from other bHLH proteins and indicate that they have distinct biological functions. The purpose of this dissertation is to elucidate the oncogenic roles of DEC1 and determine the molecular actions of DEC1 on transcription regulation.

Expression measurements demonstrate that DEC1 is abundantly expressed in cancer tissues but not in adjacent normal tissues. In stable transfectants, DEC1 inhibits cell proliferation, antagonizes serum deprivation-induced apoptosis, and selectively decreases the activities of several major caspases. Western blotting analyses identify that antiapoptotic protein survivin is a functional mediator responsible for DEC1-directed antiapoptotic activity. DEC1 and survivin

exhibit a paralleled expression pattern in paired tumor-normal tissues. In co-transfection experiments, DEC1 stimulates the survivin promoter, and this mechanism relies on the physical interactions with Sp1 sites in the proximal promoter. In contrast, DEC1 and its structurally related protein DEC2 show an inverted expression pattern in paired tumor-normal tissues. Forced expression of DEC1 causes proportional decreases in the expression of DEC2 in stable transfectants. Co-transfection with DEC1 represses the activity of a DEC2 promoter reporter by as much as 90%. The DEC1 mediated transcription repression is achieved by direct binding to the E-box element in the proximal promoter of DEC2. The established activation of the survivin promoter provides a molecular explanation for its oncogenic involvement, and the differential activities toward DEC2 and survivin promoters establish that DEC1 can act as a repressor or an activator depending on the genomic context of a target gene.

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INTRODUCTION

Gene expression within eukaryotic cells is primarily regulated at transcription level. The transcription of genes is controlled by complex interactions between transcription factors and their corresponding DNA sequences in target genes. Transcription factors have two functional domains: one for sequence-specific DNA binding and one for transcription activation (Brivanlou et al. 2002). In some cases this dual requirement is shared between partner proteins, hence the site-specific DNA binding domain and transcription activation domain occur on separate proteins. Based on the structural features of the DNA binding domain, transcription factors fall into four major structural families: (1) Helix-turn-helix; (2) Zinc finger; (3) Leucine zipper; and (4) Helix-loop-helix.

Helix-loop-helix transcription factors

Helix-loop-helix (HLH) transcription factors play important roles in a wide array of developmental processes such as myogenesis (MyoD/E47) (Weintraub et al, 1991; Zhuang et al, 1994), neurogenesis (NeuroD, Ashaete-scute/Daughterless) (Villares et al, 1987; Caudy et al, 1988), hematopoiesis and sex determination (E12/Da/ESC) (Chen et al, 1990; Benton et al, 1993). Other functions of HLH proteins include regulation of cell proliferation and apoptosis (Myc/Max) (Morgenbesser et al, 1995; Bissonnette et al, 1994), phospholipid/pigment metabolism (Ino2/Delila) (Zhao et al, 1993; Goodrich et al, 1992) and xenobiotic response (AHR/ARNT) (Hirose et al, 1996; Burbach et al, 1992). HLH proteins are divided into two broad functional groups based upon their tissue distributions (Murre et al, 1994, 1992). Class I

HLH proteins, also known as E proteins like E12 and E47, have a ubiquitous expression pattern and are capable of forming either homo- or hetero-dimers. Class II HLH proteins such as MyoD and NeuroD exhibit a tissue-restricted expression pattern and usually form hetero-dimers with class I HLH proteins. Other classification systems have also been proposed based on the structural domains or evolutionary relationship (Atchley and Fitch, 1997; Massari and Murre, 2000).

1. Structure and DNA binding activity of HLH proteins

Structurally, all helix-loop-helix proteins possess a HLH motif which comprises two highly conserved helical segments (helix 1 and helix 2) of hydrophobic residues separated by a non-conserved sequence of variable length (the loop). The loop contains a stretch of amino acids unfavorable for helix formation. The HLH domain is usually adjacent to a short region of basic residues that constitute the sequence-specific DNA binding interface (Ellenberger, 1994). Therefore, Helix-loop-helix proteins are also referred to as basic helix-loop-helix (bHLH) proteins. X-ray structures of many bHLH proteins have confirmed the belief that bHLH proteins bind DNA as dimers in which the conserved HLH domain mediates dimerization and the basic region defines the DNA binding sequence (Ferre et al. 1993,1994; Ma et al. 1994). The basic region, immediately N-terminal to the first helix, consists of a cluster of 10-20 hydrophilic residues rich in lysine and arginine and binds to the major groove of the DNA in the X-ray structure.

The response DNA element recognized by bHLH proteins is usually short palindromic sequence CANNTG where N represents any base, most commonly either CG or GC. CANNTG, also known as E-box, is found in promoter and enhancer elements that regulate large numbers of pancreatic-, lymphoid- and muscle-specific genes (littlewood and Evan, 1998). As shown by X-ray structure, each monomer of the bHLH protein binds one half-site of the E-box. Oligonucleotides flanking the core E-box sequence also contribute, at least in part, to the specific interactions between the E-box and bHLH proteins. Although most of bHLH proteins are able to bind the E-box, there are some exceptions. Some bHLH proteins that have proline-containing basic regions have higher affinity toward another hexamer core sequence, CACNAG, also known as N-box (Akazawa et al, 1992). AHR-ARNT complex, known to activate xenobiotic gene transcriptions, usually binds to the dioxin response element TNGCGTG. (Wu and whitlock, 1993; Basci et al, 1995).

2. Transcription regulation by HLH proteins.

After binding to DNA, the HLH protein can either activate or repress transcription.

1) Activation of transcription.

A prototypical example of transcription activator is the E protein, including E2A (E47 and E12), E2-2, and HEB (Henthorn et al, 1990). As described previously, E proteins are ubiquitously expressed and capable of forming homo-dimers or hetero-dimers with class II HLH proteins. Two transactivation domains, AD1 and AD2, have been mapped to the N-terminal regions in

E2A and HEB proteins (Aronheim et al, 1993; Massari et al, 1996). Amino acid substitutions in conserved hydrophobic residues within these motifs abolish transactivation activity. Removal of the N-terminal transactivation domains of E2A abrogates its inducing ability on B-lineage-specific gene expressions in macrophage cells (Kee and Murre, 1998). Recent reports have demonstrated that transcription coactivators, p300 and/or CBP, interact with E proteins when bound to DNA (Eckner et al, 1996). The recruitment of p300/CBP is mediated by either direct interaction (Figure 1 A) or indirect interaction. (Figure 1 B). P300/CBP have been shown to contain histone acetyltransferase (HAT), an enzyme involved in chromatin modification (Bannister and Kouzarides, 1996).

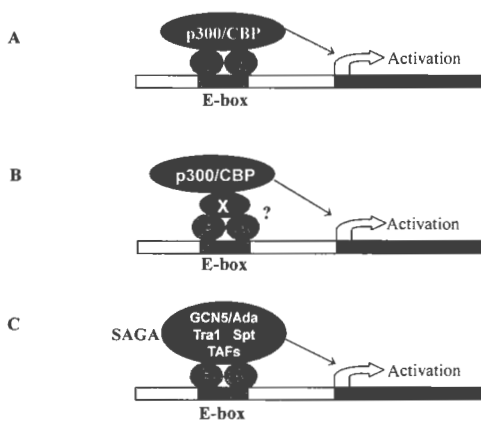


Figure 1. Models for transcription activation by E proteins

More recently, studies reports that LDFS, a conserved motif located in the AD1 domain in E protein, is capable of interacting with a nuclear protein complex, termed SAGA (Figure 1 C) (Massari et al, 1999). SAGA has intrinsic HAT activity. The components of SAGA are highly conserved from yeast to mammals. This includes a subset of the TATA binding protein-associated factors (TAFs), the HAT Gcn5, the Ada and Spt proteins, and Tral (Grant et al, 1998a, 1998b; Ogryzko et al, 1998). Amino acid substitutions of the conserved residues within AD1 domain abolish the SAGA binding *in vitro* and transcription activation *in vivo* (Massari et al, 1999). Moreover, AD1 lost the transactivation activity in yeast strains lacking functional SAGA complex. Both p300/CBP and SAGA complex have HAT activities. Transcription activation in eukaryotes is strongly promoted by histone acetylation. Hence, the E proteins activate transcription through interacting with protein complexes that have HAT activity.

2) Repression of transcription.

Over the last few years, considerable progress has been made toward understanding the repressive activities of *Drosophila* hairy and Enhancer-of-split proteins (Fisher and Caudy, 1998; Parkhurst, 1998). These proteins contain a tandem arrangement of the bHLH domain and an adjacent sequence known as the Orange domain, so we refer to these proteins as bHLH-Orange or bHLH-O proteins. The hairy-related proteins contain proline residue within their basic domain and prefer binding to N-box as a homo-dimer or hetero-dimer. Once bound

to DNA, these proteins recruit a co-repressor known as Groucho (Figure2 A). The interaction with Groucho is mediated through a 4-amino acid motif, WRPW (Trp-Arg-Pro-Trp), present at the C terminus of all hairy-related proteins. A more recent study demonstrates that Groucho can functionally interact with Rpd3 (Chen, 1999), a histone deacetylase (HDAC). This finding raises the possible mechanism that Groucho represses the transcription through recruiting chromatin modification enzyme.

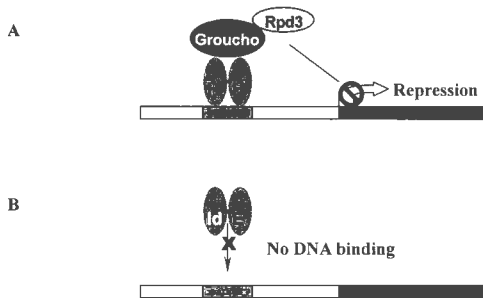


Figure 2. Models for transcription repression by HLH proteins

Another example of negative regulation on transcription by HLH protein is dominant negative effect. Id and emc proteins represent a class of HLH proteins that have distinct structural features (Benezra et al, 1990; Ellis et al, 1990). These proteins contain a HLH domain but lack the adjacent basic domain necessary for DNA binding . Heterodimerization with these proteins results in a HLH dimer that is incapable of binding to DNA (Figure2 B). Thus far, four Id

proteins (Id1 to Id4) have been identified. All of them share a homologous HLH region and have similar affinities to various E proteins (Langlands et al, 1997). E proteins play important roles in cell differentiation and activate transcription through homo or hetero-dimer formation. Id proteins dimerize with E proteins and attenuate their function as transcription activators. For example, Id1 inhibits muscle differentiation by dimerizing with E12 protein and prevent it from forming active myogenic MyoD/E12 hetero-dimer (Neuhold and Wold, 1993).

3. Dimerization partners are important for the functions of HLH proteins

Given the fact that some HLH proteins are active as hetero-dimers, the availability of each dimerization partner is clearly an important factor in dictating which particular dimer will form. Although some HLH proteins (like E proteins) are able to form functional homo-dimers, their activities can be modulated through hetero-dimerization with other partners. Consistent with this, many HLH proteins show strict spatial and temporal expressions during cell differentiation. Therefore, patterns of expression of HLH proteins are critical in maintenance of cell lineages and decision of cell fates.

Myc/Max/Mad proteins give a good example in understanding how HLH proteins exhibit their biological activities through dimerizing with different partners (Figure 3). The Myc family of proteins has been shown to regulate a wide range of processes, including oncogenic transformation, apoptosis and cell cycle progression. Previous studies indicate that c-Myc

protein level correlates with cell proliferative state (Evan and Littlewood, 1993). Thus, c-Myc is always expressed in mature, proliferative cells. Withdrawal of growth factors triggers rapid and synchronous down-regulation of c-Myc protein level in cells, resulting in growth arrest. The biological activity of c-Myc is through dimerizing with another bHLH protein, Max (Amati et al. 1993). The Max/Myc dimer binds to classic E-box CACGTG, leading to transcription activation. Studies demonstrate that c-Myc interacts with protein complex involved in chromatin modification. A SWI-SNF chromatin remodeling complex, hSNF5(Ini1), interacts with the bHLH-LZ domain of c-Myc (Cheng et al, 1999). TRAPP, related to Tra1 that is present in several HAT complexes, interacts with N terminus of c-Myc (McMahon et al, 1998). These findings suggest that c-Myc activates transcription, at least in part, by recruiting chromatin remodeling complex. c-Myc has a very short half-life of both mRNA and protein whereas Max protein is extremely stable. Max protein level is invariant throughout the cell cycles. Furthermore, Max can form homo-dimers and bind to the same E-box sequence CACGTG as Max/Myc heterodimer does. But Max homo-dimer does not show any activities on transcription regulation in vitro (Kretzner et al. 1992). Taken together, the ratio of Max/Myc versus Max/Max is thus critical to determine the Myc activity within cells.

In addition to Myc proteins, four other proteins, Mad1, Mad3, Mad4 and Mxi1, also form hetero-dimers with Max. These dimers recognize the same E-box CACGTG as Mad/Myc or Mad/Mad dimer does. Unlike Max/Myc which activates transcription, or Max/Max which is

transcription silent. Mad and Mxi1 actively repress transcription in reporter assay (Ayer et al, 1993). Mad proteins can directly interact with mammalian homologs of the yeast repressor Sin3 through the N-terminal Mad repression domain (SID) (Ayer et al, 1995). Sin is identified as a component of a protein complex that includes nuclear receptor co-repressors, N-CoR and HDAC (alland et al, 1997). Therefore, Max/Mad represses transcription through interacting with a protein complex containing HDAC activity. Mad proteins have been shown to exhibit growth inhibitory activity, which is opposite to Myc's function. Both Mad and Mxi1 are able to suppress co-transformation of rat embryo cells by c-Myc and activate ras alleles (Lahoz et al, 1994; Koskine et al, 1995). Opposite expression patterns of Mad and Myc proteins are observed in differentiated cells and tissues where Max remains relatively constant. All these data suggest that activity of genes responsible to these bHLH transcription factors can be simply modulated by changes in the ratio of Myc versus Mad (or Mxi1).

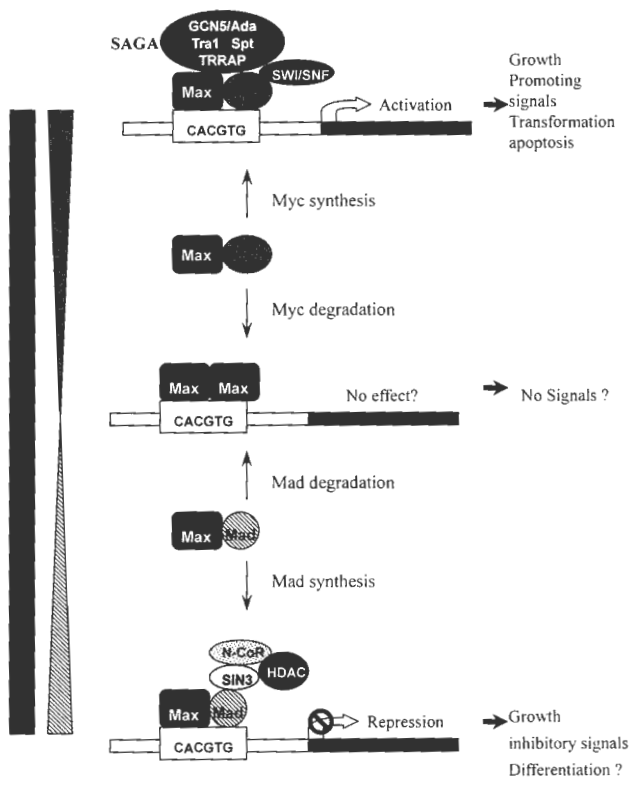


Figure 3 The Myc network

DEC/STRA/SHARP helix-loop-helix transcription factors

Human DEC1 (differentially expressed in chondrocytes), mouse STRA13 (stimulated with retinoic acid), and rat SHARP2 (split and hairy related protein) represent a structurally distinct class of bHLH proteins and are identified independently by three research groups. Shen et al (1997) identifies Bt2cAMP-inducible gene, DEC1, from primary culture of human embryonic chondrocytes. Boudjelal et al (1997) demonstrates that STRA13 is induced by stimulation of embryonal carcinoma cells (P19) with retinoic acid.

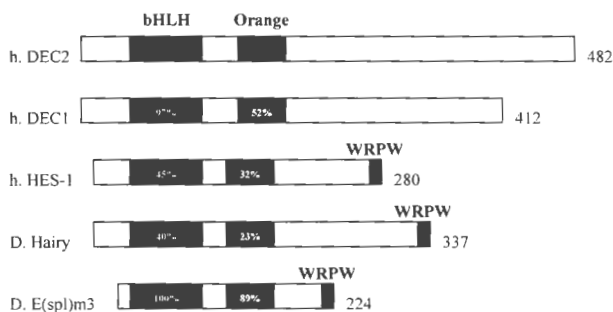


Figure 4. Schematic representation of the structures of DEC2 and related bHLH proteins. Percentages indicate amino acid identity in the bHLH region or the Orange domain between the respective proteins and DEC2. The number of amino acids are shown on the right. h. human; D. Drosophila.

Rossner et al (1997) identifies SHARP2 in an effort to identify new bHLH proteins in differentiated rat neurons and find that it is distantly related to Drosophila enhancer-of-split and hairy gene family. Human, mouse and rat DEC2 are subsequently identified by Fujimoto et al

(2001). DEC2 proteins have >90% identity to DEC1/STRA13/SHARP2 in the bHLH regions and ~40% identity in the total proteins (Figure 4).

Protein sequence alignment reveals that DEC/STRA/SHARPs show higher similarities (~40%) to *Drosophila* Hairy and E(spl) as well as the mammalian homologues (e.g., HES) in the bHLH domain (Figure 4) (Giebel et al, 1997; Poortinga et al, 1998; Fisher et al, 1996). Similar to Hairy/E(spl)/Hes, DEC/STRA/SHARPs contain an orange domain just C-terminal to the bHLH domain and a proline residue in the basic region. However, the proline residue is located 2 amino acid residues more N-terminal (Figure 5). Beside bHLH domain and orange domain,

		Basic domain			
Hes1	28	PKTASEHRKS	SKPIMEKRRR	ARINESLSOL	57
Hes2	7	VEDAADVRKN	LKPLLEKRRR	ARINESLSQL	36
Hes5	10	MLSPKEKNRL	RKPVVEKMRR	DRINSIEQL	39
E(spl)M3	5	MSKTYQYRKV	MKPLLEKRRR	ARINKCLDDL	34
E(spl)M7	7	MSKTYQYRKV	MKPLLEKRRR	ARINKCLDEL	36
Hairy	25	ETPLKSDRRS	NKPIMEKRRR	ARINCLNEL	54
Dpn	34	GLSKAELRKT	NKPIMEKRRR	ARINHCLNEL	63
DEC1	46	SEDSKETYKL	PHRLIEKKRR	DRINECIAQL	75
DEC2	48	RDDTKDITYKL	PHRLIEKKRR	DRINECIAQL	77

Figure 5. The basic regions of Hairy/E(spl)/Hes proteins and DEC proteins. The basic domains of representative proteins are aligned. The conserved residues are emphasized in bold type. Notably, proline residues in DEC proteins are located more N-terminal. The regions of the basic domains are indicated by residue numbers adjacent to the sequence.

DEC/STRA/SHARPs do not show similarity to any known proteins; especially, they lack the C-terminal WRPW motif which is present at all other Hairy/E(spl)/Hes proteins and mediates transcription repression. These structural differences distinguish DEC/STRA/SHARPs from

other bHLH proteins and indicate that they have distinct biological functions.

1. Transcriptional regulation mediated by DEC/STRA/SHARPs

Although DEC/STRA/SHARPs lack the WRPW domain which is required for transcription repression in Hairy/E(spl)/Hes proteins, they are still found to act as transcription repressors in different cell systems. STRA13 is found to repress its own promoter and c-myc promoter reporter genes (Sun and Taneja, 2000). Both repressions require the c-terminal region which interacts with HDAC-Sin3A co-repressor complex as well as basal transcription factors TATA-binding protein (TBP), TFIID and TFIIB (Boudjelal, 1997). STRA13 represses its own promoter through an HDAC-dependent pathway and can be abrogated by trichostatin (TSA), a specific HDAC inhibitor. c-Myc repression by STRA13 is HDAC-independent and involves interaction with basal transcription factor TFIIB. Thus, STRA13 represses transcription through either interacting with histone deacetylase or affecting basal transcription machinery.

Most bHLH proteins bind DNA as either hetero- or homo-dimers to E-box. Hairy/E(spl)/Hes proteins which have proline-containing basic regions prefer binding to the N-box. Like Hairy/E(spl)/Hes, DEC/STRA/SHARPs also contain a proline residue at basic region. However, several groups demonstrate that DEC/STRA/SHARPs bind to E-Box CACGTG as homo-dimers (Zawel et al, 2002; St-Pierre et al, 2002). Whether DEC/STRA/SHARPs bind to N-box remains to be clarified.

2. Expression regulation of DEC/STRA/SHARPs

Both DEC1/STRA13/SHARP2 and DEC2 are shown to be ubiquitously expressed but have different expression patterns. Many stimuli have been shown to rapidly induce the mRNA levels of DEC1/STRA13/SHARP2 within one hour in different cultured cell systems. These stimuli include: nerve growth factor (NGF), kainic acid (Rossner et al, 1997), platelet-derived growth factor (PDGF), transforming growth factor- β (TGF- β) (Zawel et al, 2002), parathyroid hormone (PTH), and cyclic adenosine monophosphate (cAMP) (Shen et al, 2001, 2002). Most of these hormones and growth factors modulate proliferation and differentiation via changes of the intracellular cAMP, a secondary messenger. Furthermore, Teramoto et al (2001) located two consensus sequences for the cAMP-responsive element in the promoter region of DEC1 gene. All these findings suggest that DEC1/STRA13/SHARP2 is a direct target of cAMP in a wide types of cells. Some other stimuli, like retinoic acid (Boudjelal et al, 1997), TSA and serum starvation, start to increase DEC1 expression at 6 hours and the high level of DEC1 is sustained for more than 24 hours. This data indicated that there might be a different signaling pathway responsible for DEC1 induction.

Another pathway that regulates the transcription of DEC/STRA/SHARP proteins is through hypoxia-inducible factor-1 (HIF-1), a transcription activator. The induction of DEC/STRA/SHARP proteins by hypoxia has been demonstrated by many groups (Ivanova et al, 2001; Yun et al, 2002). HIF-1, usually exists as an hetero-dimer of HIF-1 α and HIF-1 β , is a key

player in regulating O₂ homeostasis. Under normoxia, HIF-1 α interacts with von Hippel-Lindau tumor suppressor protein (pVHL) and undergoes proteasome mediated degradation. Under hypoxia, HIF-1 α becomes stabilized and translocated into nucleus, where it heterodimerizes with HIF-1 β and binds to hypoxia response element (HRE) to initiate transcription of target genes. Functional HREs that bind to HIF-1 α in electrophoretic mobility shift assay are found in the promoter regions of both DEC1 and DEC2 (Miyazaki et al, 2002). Disruption of these HREs abrogates the induction activity of HIF-1 α in reporter assay. Moreover, DEC1/STRA13 is shown to be down-regulated by pVHL (Ivanova et al, 2001). The increase of DEC1/STRA13 mRNA occurs in wild-type MEF cells but not in HIF-1 α ^{-/-} MEF cells. All these data demonstrate that DEC1/STRA13 is the direct target gene regulated by HIF-1 α .

In addition to transcription regulation, STRA13 is also found to be regulated by protein degradation (Ivanova et al, 2001). The yeast two-hybrid screening reveals that human ubiquitin-conjugating enzyme (UBC9) interacts with the c-terminus of STRA13 protein. Co-expression of STRA13 and UBC9 increases ubiquitination of STRA13 and shortens its half-life. Proteasome inhibitor treatment accumulates STRA13 and blocks its degradation. These results suggest that STRA13 is degraded through ubiquitin-dependent proteasome pathway.

Overall, the regulation of DEC/STRA/SHARP proteins is either through transcription activation by cAMP and HIF-1 α or through protein degradation pathway associated with UBC9/ubiquitin.

3. Biological functions

Although DEC/STRA/SHARP proteins are regulated by hypoxia and some environmental stimuli, their biological roles are poorly defined. Distinct tissue distribution patterns indicate that these proteins are important in cell differentiation. In P19 embryonal carcinoma cell lines, overexpression of STRA13 results in neuronal differentiation, while wild-type P19 cells only undergo mesodermal/endodermal differentiation (Boudjelal et al, 1997). Two rat SHARP proteins have been shown to play roles in terminal neuronal differentiation and adaptive changes to environmental stimuli (Rossner et al, 1997). Human DEC1 is found to promote chondrocyte differentiation both at the early and terminal stages (Shen et al, 2002).

In addition to neuronal and chondrocyte differentiation, STRA13 has been found to regulate lymphocyte activation. STRA13 is highly expressed in unstimulated, resting B cells and is strongly repressed during B cells activation as well as a variety of stimuli that activate B cells (Seimiya et al, 2002). Forced expression of STRA13 in B cells delays the cell cycle progression into S phase. This data suggests that STRA13 is a negative regulator of B cell activation by affecting cell cycle progression. In contrast, STRA13 plays a different role in T cell

development (Sun et al, 2001). Up-regulation of STRA13 is found upon activation of CD4+ T cells. STRA13 knockout mice exhibit defects in several phases of T cell activation. These defects include reduced cytokine production like IL-2, IFN- γ and IL-4; impaired differentiation of T cells into effector cells; and ineffective elimination of activated T and B cells.

More recently, DEC1 and DEC2 have been found to function as regulators of the mammalian molecular clock (Honma et al, 2002). The circadian rhythms of mammals are regulated by four clock-gene families that form a transcription-translation feedback loop. Clock and Bmal1 bind to the E-box elements in the promoter region of Per as a hetero-dimer and activate its transcription. The Per protein, in turn, acts with Cry protein and represses its own transcription. Like all these four gene families, DEC1 and DEC2 are expressed in the suprachiasmatic nucleus of the hypothalamus in a circadian fashion. Gel mobility shift assay and luciferase assay demonstrated that DEC1 and DEC2 bind to the proximal E-box elements in Per promoter and strongly repress the transcription of Per. Yeast two-hybrid assay reveals that both DEC1 and DEC2 bind stereo-specifically to Bmal1. Thus, DEC proteins repress the Per transcription through competition for E-box with Clock/Bmal1 and/or interaction with Bmal1.

STATEMENT OF PURPOSE

The basic helix-loop-helix (bHLH) proteins belong to a family of well-characterized transcription factors that are involved in cell differentiation, xenobiotic response and oncogenesis. Human DEC, mouse STRA and rat SHARP represent a new and structurally distinct class of bHLH proteins. In each species, two related members are identified with sequence identity of >90% in the bHLH regions and ~40% in the total proteins. The bHLH proteins contain two functional domains: helix-loop-helix (HLH) domain and the adjacent basic domain. The HLH domain is responsible for the dimerization whereas the positively charged basic region mediates sequence-specific DNA binding. Based on the sequence alignment, DEC/STRA/SHARP proteins closely resemble *Drosophila* Hairy and E(spl) as well as the mammalian homologues (e.g., HES). In addition to the bHLH domain, these proteins contain an orange domain which is approximate 30 amino acids closer to the C-terminus and is thought to mediate transcription repression. Beside bHLH domain and orange domain, DEC/STRA/SHARP proteins do not show similarity to any known proteins. Especially, they lack the C-terminal WRPW domain which is present at all other Hairy/E(Spl)/Hes proteins and recruits the transcription co-repressor Groucho. These structural differences indicate that DEC/STRA/SHARP may have distinct biological functions and unique mechanisms on transcription regulation.

In an effort to identify genes that are differentially expressed in colon carcinoma and the adjacent normal tissue, we performed cDNA subtraction assays and found that DEC1 gene was

abundantly expressed in colon carcinoma but not in the adjacent normal tissue. High expression level of DEC1 is not only observed in colon carcinoma tissue, but in many other cancer tissues as well. Some detrimental conditions, like hypoxia and treatment with cytotoxic stimuli, markedly induce the expression of DEC1 in many cell lines. Cells that lack the functional tumor suppressor gene VHL (Von Hippel-Lindau) express higher levels of DEC1. All these observations suggest that DEC1 is involved in oncogenesis. However, little is known about the functions of DEC1 in oncogenesis. The studies performed in manuscript I aimed to determine whether DEC1 plays a role in oncogenesis. To test this possibility, stable transfectants were prepared in which DEC1 can be inducibly expressed by tetracycline. These stable transfectants are used to test the effects of DEC1 on proliferation and apoptosis, two events involved in oncogenesis. The studies described in manuscript II aim to extend the studies of manuscript I and to determine the signaling pathways that lead to antagonism of apoptosis.

Transcription regulation by the bHLH proteins is primarily mediated by binding to *cis*-acting DNA elements present in the target genes via the basic region in the bHLH sequence. These DNA elements contain core sequences CANNTG or CANNAG, which are commonly referred to as E-box or N-box, respectively. Generally, proline-containing basic regions have higher affinity toward the N-box whereas the basic regions without a proline preferentially recognize the E-box. DEC1 contains a proline residue at its basic domain. A recent study shows that mouse STRA13 binds to typical E-box sequence CACGTG. DEC1 and DEC2 have been shown

to have different tissue distributions. E-box elements are located in the promoter regions of both DEC1 and DEC2. So we hypothesized that DEC1 and DEC2 are mutually regulated. The studies performed on manuscript III are to determine the molecular actions of DEC1 on the transcription regulation of DEC2. To test this, a genomic fragment of the DEC2 promoter which harbors two proximal E-box elements is inserted into a luciferase reporter. Deletion and site-directed substitution mutants of both DEC2 reporters and DEC1 constructs are generated to test the requirement of E-box and structural domains of DEC1 on transcription regulation of DEC2. Electrophoretic mobility shift assay is used to test the DNA-protein interactions.

In general, the studies described in this dissertation establish the oncogenic roles of DEC1 and elucidate the signaling pathway leading to this function. The studies on transcription regulations mediated by DEC1 provide molecular mechanisms that expand our basic understandings of DEC1 functioning as a transcription factor.

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MANUSCRIPT I

DEC1/STRA13/SHARP2 IS ABUNDANTLY EXPRESSED IN COLON
CARCINOMA, ANTAGONIZES SERUM DEPRIVATION INDUCED APOPTOSIS AND
SELECTIVELY INHIBITS
THE ACTIVATION OF PROCASPASES

ABSTRACT

The basic helix-loop-helix (bHLH) proteins are intimately associated with developmental events such as cell differentiation and lineage commitment. The HLH domain in the bHLH motif is responsible for dimerization whereas the basic region mediates DNA binding. Based on sequence alignment and domain analysis, DEC/STRA/SHARPs represent a new class of bHLH proteins. This report describes the functional characterization of DEC1 (differentially expressed in chondrocytes). Subtractive experiments and blotting analyses demonstrated that DEC1 was highly expressed in colon carcinomas but not in the adjacent normal tissues. Several cell cycle blockers markedly induced DEC1 expression. Stable transfectants with a tetracycline inducible construct demonstrated that DEC1 caused proliferation inhibition, antagonized serum deprivation-induced apoptosis, and selectively inhibited the activation of procaspases. These activities were highly correlated with the abundance of tetracycline-induced DEC1. Stable transfectants expressing a mutant DEC1 (lacking the DNA binding domain) exhibited neither proliferation inhibition nor apoptotic antagonism, suggesting that DNA binding is required for these actions. Enzymatic assays and

immunoblotting analyses demonstrated that induction of DEC1 by tetracycline markedly decreased the activation of procaspase 3, 7, and 9 but not 8. The selective suppression on the activation of pro-caspases 3, 7 and 9 over 8 suggests that DEC1-mediated antiapoptosis is achieved by blocking apoptotic pathways initiated through the mitochondria. The data functionally distinguish DEC1 from other bHLH proteins and directly link this factor to oncogenesis.

INTRODUCTION

The basic helix-loop-helix (bHLH) proteins belong to a family of transcription factors (Littlewood and Evan, 1998). These proteins are known to play important roles in myogenesis (MyoD/E47) (Weintraub et al, 1991), neurogenesis (NeuroD) (Villares and Cabrera, 1987), cell proliferation and differentiation (Myc/Max) (Bissonnette et al, 1994), sex determination (E12/Da/ESC) (Cronmiller et al, 1988), regulation of immunoglobulin genes (TFEC/TFE3) (Beckmann et al, 1990), phospholipid metabolism (Ino2/Delila) (Nikoloff et al, 1992) and xenobiotic response (AHR/ARNT) (Hirose et al, 1996). The bHLH proteins are structurally featured by their bHLH motif (Littlewood and Evan, 1998). The bHLH motif is divided into two functional domains: the helix-loop-helix (HLH) domain and an adjacent basic region. The HLH domain, consisting of two-amphipathic α -helices connected by a nonconserved loop of a varying length, is responsible for the dimerization between HLH proteins. The basic region, just N-terminal to the first helix, consists of a cluster of 10-20 amino acids rich in lysine and arginine residues and mediates sequence-specific DNA binding (Littlewood and Evan, 1998; Massari and Murre, 2000; Murre et al, 1994).

Human DEC, mouse STRA and rat SHARP represent a new and structurally distinct class of bHLH proteins (Boudjelal et al, 1997; Fujimoto et al, 2001; Rossner et al, 1997; Shen et al, 1997). In each species, two related members are identified with a sequence identity of >90% in the bHLH region and ~50% in total. Based on sequence alignment, DEC/STRA/SHARPs

closely resemble *Drosophila* Hairy and E(spl) as well as the mammalian homologues (e.g., HES) (Fisher et al, 1996; Biebel and Campos-Ortega, 1997; Poortinga et al, 1998). They share the highest sequence identity (~40%) in the bHLH domain. The basic region contains a proline residue usually located in repressive bHLH proteins (Littlewood and Evan, 1998). Like Hairy/E(spl)/Hes, DEC/STRA/SHARPs contain an orange domain, which is approximate 30 amino acids away from the second helix of the bHLH motif and is thought to mediate local repression of bHLH activators. In contrast to Hairy/E(Spl)/Hes proteins, DEC/STRA/SHARPs lack the C-terminal WRPW motif. Through this sequence, Hairy/E(spl)/Hes recruit a corepressor known as Groucho to the transcription regulatory complex (Fisher et al, 1996; Chen et al, 1999). Recruitment of Groucho is responsible for a vast array of biological activities of Hairy/E(spl)/Hes proteins including cellular differentiation and lineage commitment (Fisher et al, 1996; Chen et al, 1999; Hojo et al, 2000; Ohtsuka et al, 2001).

Lack of the functionally important WRPW motif suggests that DEC/STRA/SHARPs have rather distinct biological functions and do so through different mechanisms. In a cultured cell system, mouse STRA13 has been shown to promote neuronal but repress mesodermal and endodermal differentiation (Boudjelal et al, 1997). NIH 3T3 cells transfected with STRA13 exhibit a reduction in colony forming numbers compared with cells transfected with an empty vector (Sun and Taneja, 2000). Disruption of STRA13 gene results in defective T cell activation and the genesis of autoimmune disorders in aging mice (Sun et al, 2001). Two rat

SHARP proteins have distinct tissue distributions but both are present in a subset of mature neurons and drastically induced by neurotrophins and glutamatergic neurotransmission (Rossner et al, 1997), suggesting that the rat proteins are involved in the regulation of terminal differentiation and in the adaptive changes to environmental stimuli. Both mouse STRA13 and rat SHARP1 are shown to cause transcriptional repression (Sun and Taneja, 2000; Garriga-Canut et al, 2001). Human DEC1 is present in a wide range of tissues including some of tumor tissues and tumor-derived cell lines (Fujimoto et al. 2001; Shen et al, 1997; Ivanova et al, 2001; Zawel et al, 2002). Absence of the von Hippel-Lindau tumor suppressor proteins up-regulates the expression of DEC1 gene (Ivanova et al, 2001). These findings suggest that DEC1 has both physiological and oncogenic significance.

This report describes the functional characterization of DEC1. Subtractive experiments with matched samples demonstrate that DEC1 is highly expressed in colon carcinomas but not in the adjacent normal tissues. DEC1 expression is markedly induced by several growth arrest factors. Stable transfectants demonstrate that DEC1 causes a nutrient-dependent proliferation inhibition, antagonizes serum deprivation-induced apoptosis and selectively inhibits the activation of procaspases. In contrast, stable transfectants expressing a mutant DEC1 (lacking the DNA binding domain) show neither proliferation inhibition nor apoptotic antagonism, suggesting that DNA binding is required for these actions. These findings functionally characterize DEC1 and directly link this factor to oncogenesis.

MATERIALS AND METHODS

Chemicals and supplies

Hydroxyurea, nocodazole and amino acid deficient media were purchased from Sigma. The goat anti-rabbit-IgG conjugated with alkaline phosphatase or horseradish peroxidase and ECL substrate were from Pierce. Substrates for caspases were from Biomol. Antibodies recognizing cleaved procaspases were from Cell Signaling. DMEM medium and PCR reagents were from Gibco. Unless otherwise indicated, all other reagents were purchased from Fisher Scientific.

Plasmid

A cDNA encoding the full-length DEC1 was isolated by a cDNA-trapping method (Hu and Yan, 1999). In order to establish stable transfectants, several DEC1 constructs were prepared with the tetracycline inducible pcDNA4/TO vector. These constructs included DEC1-sense, DEC1-antisense and DEC1-mutant. The constructs were prepared by a PCR-based method with the DEC1 cDNA as the template. The primers used for PCR amplification were extended to include appropriate endonuclease sites to facilitate cloning. DEC1-sense insert (the entire coding sequence) was generated with primers S-1 (ccggaattcATCCAGACGCTCCGCTAGTG) and A-1 (ctgctetagaGCAGGAAGTAGCGAGGAAGG) whereas DEC1-antisense insert was generated with primers S-1 and A-2 (acttaagcttGGTAGGAAGTAGCGAGGAAGG). Primers A-1 and A-2 contained the same sequence but different endonuclease sites, which enabled the

PCR-generated fragments to be ligated into the vector in sense or antisense orientation. DEC1-mutant insert was generated by two steps of PCR. The N-terminal fragment was generated with primers S-1 and A-3 (CTCGTTAATCCGGTCACGCTCCTTGCTCTCGCTC-CG) whereas the C-terminal fragment was generated with primers S-2 (CGGAGCGAGAGC-AAGGAGCGTGACCGGATTAACGAG) and A-1. These two fragments were then annealed through the fusion primers A-3 and S-2, and the missing strands were subsequently filled. The resultant sequence, lacking the region encoding the DNA binding domain, was then amplified again with primers S-1 and A-1, and subcloned into the pcDNA4/TO vector. The resultant constructs were subjected to sequencing analyses.

Tissue collection and processing

Samples were collected from patients who underwent subtotal colon resection for histologically confirmed colonic adenocarcinoma. A total of four samples were collected. The age of the patients was between 41 and 62 with two male and two female. The size of tumors was ~5 cm in diameter and located at the sigmoid or left colon. The degree of differentiation of tumors was moderate as determined by pathological examination. Samples were freshly processed for RNA isolation and protein extraction. Total RNA was isolated with a Tri-reagent as described previously (Zhang et al, 1999). For the preparation of protein extracts, tissues were homogenized in lysis buffer (20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.2 mM PMSF and 1 mM DTT). The homogenates were centrifuged at

12,000 g for 30 min to remove any insoluble precipitates. As controls, specimens from the adjacent, grossly normal tissues were similarly processed. Use of human tissues was approved by the Institutional Review Board.

PCR-select cDNA subtraction

Differential expression of genes between colon carcinomas and the adjacent normal tissues was studied by PCR-select cDNA subtraction with a PCR-select cDNA kit (Clontech). PolyA(+) RNA was isolated by two cycles of oligo(dT)-cellulose column chromatography. The polyA(+) RNA from four individual cancer tissues was pooled, so was the polyA(+) RNA from the adjacent normal tissues. First strand cDNA was synthesized with AMV reverse transcriptase and the second-strand synthesis was completed with RNase H and DNA polymerase I. The collection of cDNAs from both tester (cancer) and driver (normal) was blunted by Rsa I digestion. The tester cDNAs (tumor tissue) were then divided into two portions, which were ligated to adaptor 1 and adaptor 2R, respectively. Tester cDNAs ligated to each adaptor were separately mixed with the driver cDNAs to remove sequences present in both tumor and normal tissues. The reactions were then combined to allow remaining single stranded cDNAs to hybridize. The hybridized duplexes with one strand having adaptor 1 and the other having adaptor 2 represented the sequences with a higher abundance in tumor tissue than the adjacent normal tissues because only the cDNAs from the tumor tissues were ligated to these adaptors. After filling the missing strand of the adaptors, these sequences were amplified by PCR with

nested primers, which were designed to effectively amplify the hetero-adaptor sequences. The enriched, differentially expressed sequences were purified and ligated into the pTarget vector (Promega). The resultant clones were isolated and subjected to sequencing analysis.

Subcellular localization of DEC1

Cells (human embryonic kidney 293T) were plated in 35 mm culture dish in DMEM medium supplemented with 10% fetal calf serum at a density of 3×10^5 cells per dish. On day 2 when the cells reached ~70% confluence, cells were transfected with DEC1 or the corresponding empty plasmid for 4 hr with Lipofectamine (Gibco). Thereafter, the media were replaced with fresh media. The transfected cells were cultured for an additional 48 hr. Cells were washed once with PBS and scraped from the dish in PBS. Cells were pelleted by centrifugation at 3000 g and rapidly resuspended in three pellet volumes of homogenization buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl). The resuspended cells were then subjected to a glass Dounce homogenizer for ten strokes. The cytoplasm and nucleus were separated by centrifugation at 3,000 g for 15 min, and the nuclear pellets were resuspended in lysis buffer (homogenization buffer plus 0.5% Triton-100 and 0.5% SDS). Cell fractions were analyzed for DEC1 abundance.

Stable transfection

Stable transfection was conducted with T-Rex-293 cell line, which was derived from the human

embryonic kidney 293 cells. This line had been stably transfected with the pcDNA6/TR plasmid, which encoded the tetracycline repressor and allowed the inserted sequence to be inducibly expressed by tetracycline. The T-Rex 293 cells were seeded at 50% confluence and cultured overnight. Transfection was conducted with DEC1-sense, DEC1-antisense or DEC1-mutant (1 µg) for 4 hr by Lipofectamine. Transfected cells were cultured in full media for 24 hr, then split into fresh media. The split cells were seeded to 10% confluence and cultured in media containing both Zeocin™ (300 µg/ml) and blasticidin (5 µg/ml) to select stable transfectants of pcDNA4/TO-DEC1 and maintain the integration of the pcDNA6/TR construct, respectively. Positive foci resistant to both antibiotics were picked up and expanded. The induced expression of DEC1 was determined by Western analyses.

Proliferation assay

Stable transfectants expressing sense, antisense or mutant DEC1 were seeded in a 96-well plate (12,500/per well) and cultured in DMEM media supplemented with fetal bovine serum (0-10%) in the presence or absence of tetracycline (0-1 µg/ml). After an incubation at 37°C for 48 hr in a humidified atmosphere with 5% CO₂, the proliferation rate was determined with the CellTiter kit (Promega) by measuring formazan formation converted from tetrazolium at 570 nm. The background value of the seeding cells was also determined and subtracted.

DNA Fragmentation

Stable transfectants (~80% confluence) were subjected to apoptotic induction by culturing in serum-free media in the presence or absence of tetracycline (1 µg/ml). After a 48-hr incubation, cells (1×10^6) were collected, washed with PBS and lysed with lysis buffer (5 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.5% Triton-100, 0.1% SDS). The high molecular weight DNA was removed by centrifugation at 14,000 g for 30 min. The supernatants were then sequentially extracted with equal volumes of a mix of phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform. The soluble DNA was precipitated with 2.2 volumes of ethanol in the presence of 0.5 M NaCl. The DNA was resuspended with TE and treated with DNase-free RNase (0.1 µg/µl) for 3 hr at 30°C to remove RNA. DNA fragmentation was analyzed by agarose electrophoresis (1.5%). Cell lysates were also prepared and analyzed for the expression of DEC1. In order to quantitatively specify the degree of DNA fragmentation as a function of the expression levels of DEC1, same experiments (the sense line only) were conducted but various concentrations (0-1 µg/ml) of tetracycline were added to proportionally increase the induction of DEC1.

Caspase activity

Caspase activities were determined with colorimetric substrates. Ac-DEVD-pNA, Ac-IETD-pNA and Ac-LEHD-pNA were used for caspase 3/7, 8 and 9, respectively. Stable transfectants were seeded at a ~60% confluence in 6-well plates. After cells reached ~80% confluence, the media were replaced with serum-free media and cultured in the presence or

absence of tetracycline (1 $\mu\text{g/ml}$) for 48 hr. Cells were then harvested, washed with PBS and lysed on ice for 30 min in lysis buffer (100 μl /per well). The lysis buffer contained 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5% Triton X-100, 0.1 mM PMSF, 1 mM DTT, pepstatin (10 $\mu\text{g/ml}$) and leupeptin (10 $\mu\text{g/ml}$). The lysates were then subjected to centrifugation at 10,000 g for 1 min at 4°C. To determine caspase activity, 100 μl supernatant (1 mg/ml) was mixed with 100 μl reaction buffer [20 mM Tris-HCl, pH 7.4, 4 mM EDTA, 0.2% CHAPS, 0.1 mM PMSF, 1 mM DTT, pepstatin (10 $\mu\text{g/ml}$) and leupeptin (10 $\mu\text{g/ml}$)]. To the mix, colorimetric substrate was added at a final concentration of 200 μM . After a 2-hr incubation at 37°C, the amount of released p-nitroanilide was determined by measuring the absorbance at 410 nm, and the relative activity was calculated.

Northern and Western analyses were conducted as described previously (Zhu et al, 2000; Yan et al, 1995). Preparation of an antibody against a peptide (CSQALKPIPLNLETKD) derived from DEC1 was described elsewhere (Zhu et al, 1999). Protein concentration was determined with BCA assay (Pierce). Data are presented as mean \pm SD of at least three separate experiments, except where results of blots are shown in which case a representative experiment is depicted in the figures. Comparisons between two values are made with Student's t test at a value of $P < 0.05$.

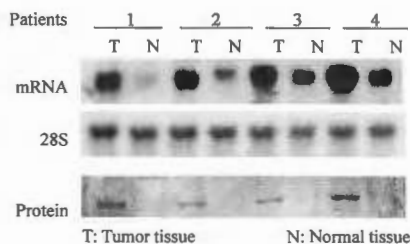
RESULTS

Molecular cloning and tumor-related expression of DEC1

In an effort to identify genes that are differentially expressed between colon carcinomas and the adjacent normal tissues, we performed cDNA subtractive experiments. Four matched samples were used. One of the genes identified encoded a bHLH protein. A full-length cDNA encoding this protein was isolated from a liver library by a cDNA-trapping procedure as described previously (Hu and Yan, 1999). The cDNA was 3,361 nucleotides long including a 179-bp polyA tail. Alignment analysis revealed that this cDNA had a 98% sequence identity with DEC1, a gene that was identified from differentiated embryo chondrocytes (Shen et al, 1997). Most of the nucleotide substitutions were in the 3' non-translation region, and none of the substitutions in the coding region caused amino acid substitutions.

We next examined the abundance of DEC1 in colon carcinomas and the adjacent normal tissues by Northern and Western analyses. As shown in Figure 1-1A, without exception, DEC1 gene was expressed abundantly in the colon adenocarcinomas, but only little or very low levels of DEC1 mRNA were detected in the adjacent normal tissues (>20 folds based on densitometrical analysis). Consistent with the mRNA data, DEC1 protein was detected only in the cancerous but not in the adjacent normal tissues. As a control for specificity of the antibody, transfection experiments were conducted. Cytoplasmic and nuclear fractions were prepared from cells transfected with DEC1 or the corresponding empty SPORT plasmid. As shown in Figure 1-1B,

A



B

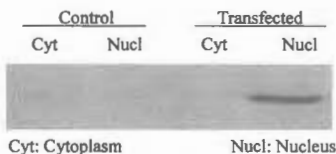


Figure 1-1. Expression of DEC1 in colon carcinoma and the adjacent normal tissues

Total RNA (10 µg) from colon carcinoma and the adjacent normal tissues was subjected to agarose electrophoresis and transferred to a Nytran nylon membrane. The blots were detected with ³²P labeled probes from cDNA encoding DEC1. To normalize the abundance of 28S rRNA, the same membrane was reprobred with a [³²P]ATP labeled oligonucleotide hybridizing 28S rRNA. For Western blotting analysis, homogenates (10 µg) from colon adenocarcinoma and the adjacent tissues were subjected to SDS-PAGE. The immunoblot was incubated with the antibody against DEC1. The primary antibody was then located by alkaline phosphatase-conjugated goat anti-rabbit IgG. (B) Specificity of the prepared antibody. The plasmids harboring the DEC1 cDNA (transfected) or no insert (control) were used to transfect 293T cells and the subcellular fractions were prepared as described in the Materials and Methods. The fractions (5 µg) were subjected to SDS-PAGE and analyzed for DEC1 expression (Right).

this antibody detected a protein with a molecular weight of ~52 kDa, which is consistent with the cDNA-based calculated molecular weight. This protein was present only in the nuclear fraction of the DEC1 transfected cells (transfected) not in the empty vector-transfected cells (control), suggesting that this antibody is highly specific and DEC1 is a nuclear protein (Figure 1-2B).

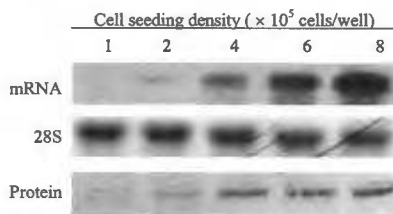
Effects of cell confluence and cycle blockers on DEC1 expression

Given the fact that DEC1 is abundantly expressed in tumor but not in the adjacent normal tissues, its expression is likely linked to cell proliferation state and cycle progression. In order to test this possibility, the expression of DEC1 was first studied as a function of cell confluence. DLD cells, a colon carcinoma-derived cell line, were seeded in a 12-well culture plate at various densities ($1-8 \times 10^5$). On day 3 when wells with the top three seeding densities were confluent or post-confluent, cells were harvested. At this time, the cells at the two lowest seeding densities were still rapidly proliferating. Total RNA and cell lysates were prepared and analyzed for the expression of DEC1. As shown in Figure 1-2A, confluent and post-confluent cells (seeded at higher densities) expressed high levels of DEC1, whereas cells in proliferative states (seeded at lower densities) expressed little DEC1. It should be emphasized that the same amount (10 μ g) of total protein was used for each sample. Consistent with the protein data, DEC1 mRNA levels were drastically elevated in the confluent and post-confluent cells.

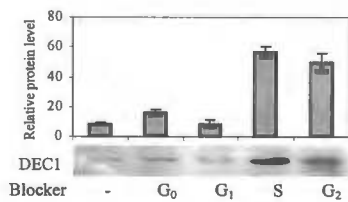
Figure 1-2. Regulation of DEC1 expression by cell cycle blockers

The abundance of DEC1 mRNA and proteins was determined by Northern and Western analyses with 10 µg of total RNA and 10 µg of cell lysates, respectively. (A) Expression of DEC1 as a function of cell confluence. DLD cells were seeded in a 12-well culture plate at various densities ($1-8 \times 10^5$). On day 3, total RNA and lysates were prepared and analyzed for the expression of DEC1. (B) Effects of cell cycle blockers on DEC1 expression. Cells were seeded at a 30% confluence. After a 12-hr incubation, media were replaced with cycle block media (G_0 block, 0.1% serum; G_1 block, amino acid deficient media; S block, 3 mM hydroxyurea; and G_2 block, 500 ng/ml nocodazole). After a 30 hr-incubation, lysates were prepared and analyzed for DEC1 expression. (C) Time course of hydroxyurea and nocodazole on DEC1 expression. Cells were treated as described in section B and harvested at indicated times, and lysates were analyzed by Western blot. Data are presented as representative results obtained in at least three independent experiments.

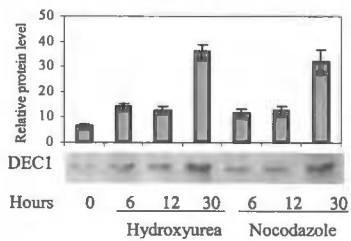
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B



C



Confluent and post-confluent cells tend to have better cell-cell contact and usually undergo growth arrest with less number of cells continuing cycle progression. Therefore, confluence-induced expression of DEC1 is likely due to cell contact, growth arrest or both. In order to test these possibilities, cells were cultured at subconfluence in the presence of cell cycle blockers. For G₀ block, cells were cultured in normal growth media but supplemented with only 0.1% serum. For G₁ block, cells were cultured in amino acid deficient media (no methionine and isoleucine). For S block, cells were cultured in normal growth media containing hydroxyurea. For G₂ block, cells were cultured in normal growth medium containing nocodazole. Cells were cultured in the cycle block media for 30 hr, and lysates (10 µg of total protein) were prepared and analyzed for the levels of DEC1. As shown in Figure 1-2B, both hydroxyurea and nocodazole were shown to markedly induce DEC1. The time course study showed that the induction was observed as early as 6 hr after treatment (Figure 1-2C) and reached the maximum 30 hr after treatment. It should be noted that similar experiments were conducted with T-Rex 293 cells. These cells expressed markedly lower levels of DEC1 and the induction was not evident (data not shown).

Cell growth rate as a function of DEC1 expression

The cell cycle arrest-related expression of DEC1 suggests that this protein slows cell cycle progress, thus, has proliferation-inhibitory effects. In order to test this possibility, constructs DEC1-sense, DEC1-antisense or DEC1-mutant (Figure 1-3A) was used to stably transfect

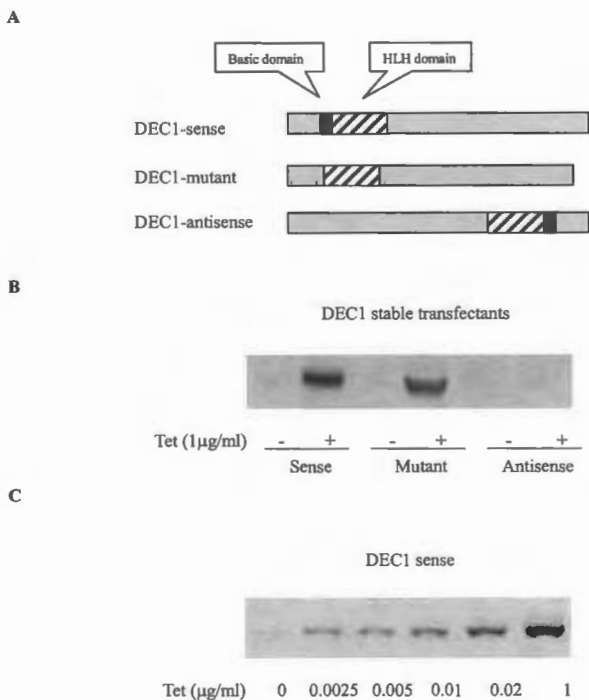


Figure 1-3. Establishment of DEC1 stable transfectants

(A) Diagrammatic presentation of constructs encoding sense, antisense and mutant DEC1. (B) Tetracycline induced expression of DEC1 in stable transfectants. The transfected cells were cultured in the presence or absence of tetracycline (Tet, 1 µg/ml) for 24 hr, and lysates were analyzed for the abundance of DEC1 (5 µg protein). (C) Concentration-dependent induction of DEC1 by tetracycline. Stable transfectant expressing the sense DEC1 was cultured in media containing tetracycline (0-1 µg/ml) for 24 hr. DEC1 induction was analyzed as described in section B. The Figure shows representative results.

T-Rex 293 cells, which allowed the inserted gene to be inducibly regulated by tetracycline. As shown in Figure 1-3B, addition of tetracycline caused a robust expression of the sense and mutant DEC1. The mutant, lacking the DNA binding domain, had a slightly faster electrophoretic mobility. No DEC1 was detected in cells transfected with the antisense construct whether or not tetracycline was added to the media. In addition, the induction by tetracycline occurred in a concentration-dependent manner (Figure 1-3C).

In order to determine the effects of DEC1 on cell proliferation, the stable transfectants expressing sense, antisense or mutant DEC1 were cultured in the presence or absence of tetracycline. After a 48 hr-incubation, the proliferation rate was determined. As shown in Figure 1-4A, the sense but not the antisense or mutant lines exhibited a significant proliferation inhibition. Such an inhibitory effect was correlated well with the amount of serum supplemented in the media (Figure 1-4B). The serum-related inhibition was more evident in cells seeded at higher densities (data not shown). Furthermore, the inhibition activity was correlated well with the abundance of induced DEC1 (Figure 1-4C).

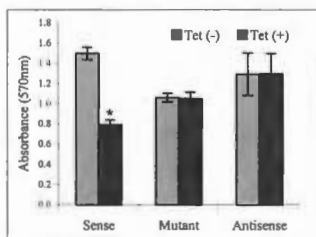
DEC1 antagonizes apoptosis during serum-deprivation

In some cases, highly proliferative cells tend to be susceptible to detrimental conditions whereas the opposite is true with quiescent or slow proliferative cells. In order to test whether DEC1 was antiapoptotic, DEC1 stable transfectants were cultured in serum-free media. After a

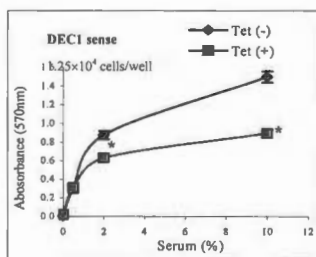
Figure 1-4. Effects of DEC1 on cell proliferation

Stable transfectants were plated in 96-well plates (12,500/well) and cultured in the presence or absence of tetracycline (Tet, 1 µg/ml) for 48 hr. The proliferation rate was determined with a CellTiter kit by measuring the absorbance at 570 nm (Promega). To precisely reflect the proliferation rate, control plates were seeded as same as experiment plates but the absorbance was determined 4 hr after seeding. Data are expressed by subtracting the absorbance from the control plates. (A) Proliferation rate of sense, antisense and mutant lines cultured with or without tetracycline (1 µg/ml). (B) Proliferation rate as a function of serum concentrations. (C) Proliferation rate as a function of tetracycline concentrations. Results were obtained in at least three independent experiments. *Significantly different from cells cultured without tetracycline.

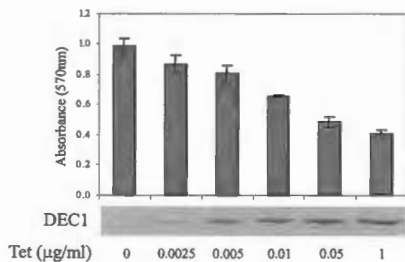
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B



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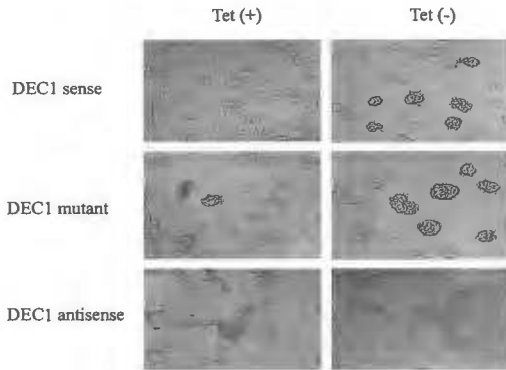
48-hr incubation, the morphological changes were studied microscopically. When cultured in the presence of tetracycline, the sense line displayed an appearance of normal cells (Figure 1-5A, top left). However, the same line, when cultured without tetracycline, underwent rapidly apoptotic changes including condensed nuclei, blebbing of plasma membrane, decreased cell size and formation of aggregates (Figure 1-5A, top right). In contrast, the mutant line underwent apoptotic changes whether or not tetracycline was added to the media (Figure 1-5A, middle). Similarly to the mutant line, the antisense line underwent a cell dying process (Figure 1-5A bottom). In contrast to the apoptotic appearance seen with the mutant line, the changes of the antisense line were associated by excess granule deposit, enlarged cell body, shrank cell processes and lack of clear cell border. In addition, the contact among the processes between two cells was markedly reduced compared with the sense line cultured in the presence of tetracycline (Figure 1-5A).

In order to link the morphological changes to DNA fragmentation, another hall-marker for cell apoptosis, stable transfectants were subjected to serum deprivation in the presence or absence of tetracycline. After a 48-hr incubation, soluble DNA was isolated and analyzed by agarose gel electrophoresis. As shown in Figure 1-5B, no DNA laddering was detected in the sense line when cultured in the presence of tetracycline. In contrast, the antisense and mutant lines or the sense line cultured without tetracycline had extensive DNA ladders. DNA laddering in the sense line was inversely correlated with the abundance of tetracycline-induced DEC1 (Figure 1-5B).

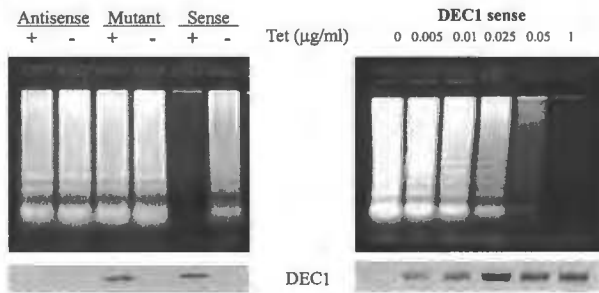
Figure 1-5. Antagonistic effects of DEC1 on serum deprivation-induced cytotoxicity

(A) Morphological assessment of DEC1-mediated protection against serum deprivation. Stable transfectants expressing sense, antisense or mutant DEC1 were plated at ~40% confluence and grown in full-media in the presence or absence of tetracycline (Tet, 1 $\mu\text{g}/\text{ml}$). After a 12 hr-incubation, the cells were then cultured in serum free media (tetracycline was kept the same). After a 48 hr-incubation, morphological changes were assessed (100 x). (B) Effects of DEC1 on DNA fragmentation in serum-deprivation induced apoptosis. Stable transfectants were grown in full-media in the presence or absence of 1 $\mu\text{g}/\text{ml}$ tetracycline until reaching ~80% confluence. The media were then replaced by serum free media (tetracycline was kept the same). After a 48 hr-incubation, DNA fragmentation was analyzed by agarose electrophoresis (1.5%). Cell lysates (5 μg) were also prepared from the same cells and analyzed for the expression of DEC1.

A



B



Selective inhibition of DEC1 on the activation of procaspases-3/7, 8 and 9

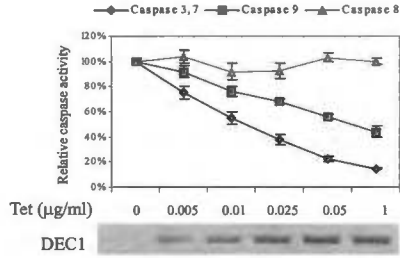
Caspases are a group of cysteinyl aspartate-specific proteases that are known to be activated during apoptotic process (Cryns and Yuan, 1998; Luscher and Eisenman, 1990). Next we examined whether expression of DEC1 caused changes on the activity of caspases-3/7, 8 and 9. Stable transfectants underwent serum deprivation in the presence or absence of tetracycline. Cell lysates were prepared and assayed for the activity of caspases-3/7, 8 and 9. As shown in Figure 1-6A, when cultured in the presence of tetracycline, the sense line showed a marked decrease on the activity of caspases-3/7 and 9 but not 8. The decrease on caspase-3/7 activity was more profound than that on caspase-9. In both cases, the decreases were inversely correlated with the abundance of induced DEC1. In contrast, the antisense and mutant lines exhibited little changes on the caspase activity whether or not tetracycline was added (data shown only on caspase-3/7) (Figure 1-6B).

Although caspases show a preference for peptidyl substrates, they are not very specific. In order to further establish the selectivity of DEC1 on the activation of several procaspases, immunoblotting analyses were performed with antibodies specific to cleaved procaspase 3, 7, 8 or 9. As shown in Figure 1-6C, when cultured in the presence of tetracycline, the sense line showed a marked decrease on the levels of cleaved procaspases 3, 7 and 9. In contrast, little changes were detected on the levels of cleaved procaspase 8. These results are consistent with the data from enzymatic assays (Figure 1-6A) and provide further evidence that DEC1

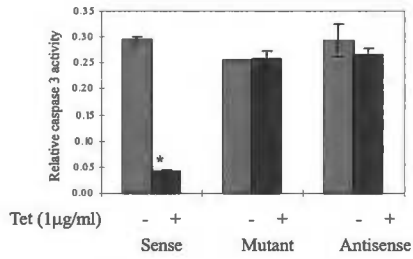
Figure 1-6. Effects of DEC1 on the activity and protein levels of caspases-3/7, 8 and 9

Stable transfectants were cultured in full media in the presence or absence of tetracycline (0-1 $\mu\text{g/ml}$) overnight and then subjected to serum deprivation for 48 hr. Lysates were prepared and assayed for caspase activity or immunoblotting analyses. (A) The dependence of suppressed activation of procaspases-3/7, 8 and 9 on the induced DEC1 in the sense line (the activity was recorded as a percentage of that when cells were cultured without tetracycline). (B) Relative activity of caspase-3/7 in stable transfectants expressing sense, antisense or mutant line upon serum deprivation. (C) Relative abundance of caspase 3, 7, 8 and 9. Lysates (50 μg) from the stable transfected sense line cultured in the absence or presence of tetracycline (1 $\mu\text{g/ml}$) during serum starvation were analyzed by Western blotting with antibodies specific to cleaved procaspase 3, 7, 8 or 9. The blots were developed by chemiluminescent substrate.

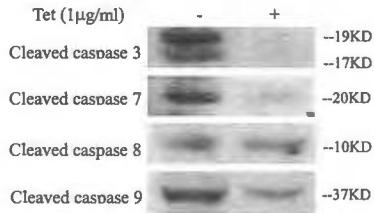
A



B



C



selectively suppresses the activation of procaspases. The selective suppression on the activation of pro-caspases 3, 7 and 9 over 8 suggests that DEC1-mediated antiapoptosis is achieved by blocking apoptotic pathways initiated through the mitochondria although the precise mechanism remains to be determined. It should be noted that little changes were observed on the expression levels of all four procaspases (inactive forms) whether or not tetracycline was added (data not shown).

DISCUSSION

Human DEC proteins, along with mouse STRA and rat SHARP, represent a new class of bHLH transcription factors (Boudjelal et al, 1997; Fujimoto et al, 2001; Rossner et al, 1997; Shen et al, 1997). This report describes the functional characterization of DEC1. Subtractive experiments with matched samples, followed by Northern and Western analyses, demonstrate that DEC1 is highly expressed in the tumor but not in the adjacent normal tissues. DEC1 expression is markedly induced by several growth arrest factors. DEC1 causes a nutrient-dependent proliferation inhibition, antagonizes serum deprivation-induced apoptosis and selectively inhibits the activation of procaspases. Stable transfectants expressing a mutant DEC1 (lacking the DNA binding domain) show neither proliferation inhibition nor apoptotic antagonism, suggesting that DNA binding is required for these actions. These findings functionally characterize DEC1 and directly link this factor to oncogenesis.

The proliferation inhibition and anti-apoptosis distinguish DEC1 from other bHLH proteins, particularly those that have oncogenic significance. The myc oncoproteins exhibit intrinsically growth-promoting activity (Luscher and Eisenman, 1990; Packham and Cleveland, 1995). Experimentally enforced c-myc expression inhibits differentiation and induces cell proliferation. Instead of suppressing apoptosis, overexpression of myc proteins induces it (Penn et al, 1990). The TAL1 protein, while absent in normal adult T lymphocytes, is constitutively expressed in >60% of T-cell acute lymphoblastic leukemia. In contrast to DEC1, ectopic

expression of TAL1 promotes cell proliferation and facilitates p53-mediated apoptosis (Condorelli et al, 1997). Twist, another bHLH protein involved in developing oncogenic phenotypes, promotes both proliferation and survival (Maestro et al, 1999). ID proteins, lacking the DNA binding domain, heterodimerize with many other bHLH proteins, therefore, act as dominant negative regulators and have a broad spectrum of biological activities (Norton, 2000). Similar to myc protein, ID proteins are proliferation-promoting and pro-apoptotic (Norton, 2000; Kim et al, 1999; Wice and Dordon, 1998).

The proliferation inhibition and antiapoptosis by DEC1 are likely to be active events through direct DNA binding. In this report, we demonstrate that only the sense but not the mutant line showed proliferation inhibition and antiapoptotic effect. The inability of the mutant to confer both activities was unlikely due to the lack of appropriate localization in the nucleus. Studies with deletion mutants located the nuclear targeting sequence of DEC1 in the C-terminal region rather than the N-terminus where the bHLH motif is present (Ivanova et al, 2001). In addition, the mutant line was shown to repress E47-mediated transcription activity toward an E-box reporter in a tetracycline-dependent manner (data not shown), suggesting that the mutant line produced biologically active proteins. Consistent with these observations, previous study with mouse STRA13 has shown that the middle section of this protein has transcription repressive activity (Boudjelal et al, 1997). The repressive activities of STRA13 are achieved through recruiting co-repressor HDAC1 or interacting with basal transcription factor TFIIB (Sun and

Taneja, 2000). These findings suggest that DEC1 exerts its biological activities through DNA-binding dependent and independent mechanisms. It should be emphasized that STRA13 and DEC1 share the DNA binding domain, however, mouse STRA13, purified or nuclear extracts from STRA13-transfected cells, has no binding activity toward any known consensus N- or E-box sequences (Boudjelal et al, 1997) whereas a GST fusion protein containing the N-terminal 122 residues of DEC1 is recently report to bind to E-box and related to sequence (Zawel et al, 2002).

Proliferation inhibition by DEC1 is consistent with the observations that DEC1 gene is up-regulated by agents that cause growth arrest. However, some cycle blockers show only minimal effects on DEC1 induction. For example, deficient medium exhibited potent inhibitory activities on cell proliferation but little changes on the levels of DEC1 were detected (Figure 1-2B). Agents with a similar cycle block activity show differential effects on DEC1 induction. For example, cAMP, hypoxia and amino acid deficient media are all known to arrest cells at G1 and delay G1/S transition. Only the first two factors were shown to drastically induce DEC1 (Ivanova et al, 2001; Shen et al, 2001, Figure 1-2B). Generally a sustained induction by cAMP, hydroxyurea and nocodazole was observed as early as 6 hr after treatment (Shen et al, 2001, Figure 1-2). Cycle synchronization normally takes ~24 hr. Such a rapid induction of DEC1 by cycle blockers suggests that the induction of DEC1 is not due to cycle arrest rather than a prior change to such an event. This notion is supported by the fact that DEC1 acts as suppressor on

cell proliferation (Figure 1-4). It should be noted that induction of DEC1 likely provides a cytoprotective role as confluent cells usually experience nutrient depletion and cell cycle blockers are generally cytotoxic.

Antiapoptotic activity of DEC1 links directly this factor to oncogenesis although it is physiologically important as well. DEC1 is expressed in a wide range of adult tissues and rat SHARPs exhibit an onset of expression as early as the end of embryonic development (E20), suggesting that DEC/STRA/SHARPs play a physiological role. In this report, we demonstrate that DEC1 was abundantly expressed in colon carcinoma but not in the adjacent normal tissues, suggesting that deregulated expression of DEC1 is responsible for its oncogenic potential. DEC1-mediated antiapoptotic action against serum deprivation is particularly relevant to the survival of tumors. Many cancer tissues particularly solid tumors are poorly vascularized, which resembles the condition of serum starvation (Naek et al, 2000; Vaupel et al, 2001; Yu et al, 2001). Poor vascularization leads to hypoxia, and survival of hypoxia is an important mechanism of tumor expansion. Interestingly hypoxia drastically induces the expression of DEC1 and the induction is abolished by von Hippel-Lindau tumor-suppressor protein (Ivanova et al, 2001), a factor that regulates activity of the hypoxia-inducible factor (HIF-1). The HIF-1 deficient tumors, although reduced vascularization, exhibit a higher expansion rate than HIF-1 expressing tumors (Naek et al, 2000; Vaupel et al, 2001; Yu et al, 2001). However, it remains to be established whether HIF-1 deficient tumors express high levels of DEC1 and whether DEC1

expression is a contributing factor to the faster expansion of HIF-1 deficient tumors.

It is interesting to notice that the stable transfectant expressing antisense DEC1 was morphologically different from the mutant line upon serum starvation (Figure 1-5A). Instead of classical apoptotic changes such as decreased cell size, the antisense line maintained the overall shape of the cells. DNA fragmentation displayed similar changes between mutant and antisense lines (Figure 1-5), suggesting that these cells undergo apoptosis to a comparable extent. The morphological difference, however, was likely due to the difference on the levels of endogenous DEC1. No endogenous expression of DEC1 was detected under the conditions employed, and the effectiveness of antisense sequence on blocking the endogenous expression, therefore, remains to be established. However, it is conceivable that the antisense line expressed less endogenous DEC1 than the sense or mutant line. It should be pointed out that the inserted DEC1 sequence was inducibly regulated by tetracycline, but the absence of this antibiotic did not completely eliminate constitutive expression of the transgene. Therefore, the endogenous DEC1 expression in the antisense line, even cultured in the absence of tetracycline, was likely to be suppressed. We tested several lines for each type of stably transfected cells and same results were consistently obtained, excluding the possibility that the difference in the location of the transgene inserted in the genome was responsible for the morphological difference upon apoptotic induction.

In conclusion, we demonstrate that DEC1 causes proliferation inhibition, antagonizes apoptosis induced by serum deprivation, and selectively inhibits the activation of procaspases 3, 7, 8 and 9. These activities are highly correlated with the abundance of tetracycline-induced DEC1. Both proliferation inhibition and antiapoptotic effect require the presence of the DNA binding domain. DEC1 is present in a wide range of adult tissues, and we demonstrate that this gene is abundantly expressed in colon carcinoma but not in the adjacent normal tissues. These findings together suggest that DEC1 is physiologically important and plays an oncogenic role when its expression is ectopic and deregulated. DEC/STRA/SHARPs are structurally distinct bHLH proteins. The data presented in this report functionally distinguish them from other bHLH proteins as well.

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MANUSCRIPT II

THE EXPRESSION OF ANTIAPOPTOTIC PROTEIN SURVIVIN IS TRANSCRIPTIONALLY UPREGULATED BY DEC1 PRIMARILY THROUGH MULTIPLE SP1 BINDING SITES IN THE PROXIMAL PROMOTER¹

ABSTRACT

Human DEC, mouse STRA and rat SHARP proteins constitute a new and structurally distinct class of the basic helix-loop-helix (bHLH) proteins. DEC1 is abundantly expressed in tumors and protects against apoptosis accompanied by decreased caspase activities. In this study, we report that DEC1 transcriptionally upregulates the expression of survivin, an antiapoptotic protein that interferes with the processing and catalysis of caspases. In paired tumor-normal tissues, survivin and DEC1 exhibited a paralleled expression pattern. Tetracycline-induced expression of transfected DEC1 caused proportional increases on the levels of survivin. In co-transfection assays, DEC1 activated a survivin promoter reporter (5-fold) but repressed a DEC2 promoter reporter (>90%). In contrast to the repression, the activation was delayed and varied depending on serum concentrations and cycle blockers. In addition, the C-terminal 65 residues were required to maximize the activation but not repression. Studies with deletion and substituted mutants located, in the proximal promoter, two Sp1 sites that supported the DEC1-mediated activity. Both DEC1 and Sp1 were involved in the binding of these Sp1 sites. DEC1 has been implicated in oncogenic process and is generally considered to be transcriptionally repressive. The established activation of the survivin promoter provides a

molecular explanation for its oncogenic involvement and represents the first example that DEC1 and other members in this class can act as transcription activators depending on the genomic context of a target gene.

INTRODUCTION

Human DEC, mouse STRA and rat SHARP constitute a new and structurally distinct class of bHLH proteins (Shen et al., 1997; Rossner et al., 1997; Boudjelal et al., 1997). In each species, two members are identified with a sequence identity of >90% in the bHLH region and ~40% in total proteins, respectively. These transcription factors are involved in various cellular events such as cell proliferation, differentiation (Boudjelal et al., 1997; Sun and Taneja, 2000), maturation of lymphocytes (Sun et al., 2001), regulation of molecular clock (Honma et al., 2002) and lipid metabolism (Yun et al., 2002). Transfection with DEC1 causes marked decreases in cell proliferation, and the decreased proliferation is proportionally correlated with the levels of DEC1 (Li et al., 2003). Chondrogenic cells expressing high levels of DEC1 undergo rapid phenotypic changes toward terminal differentiation in response to mitogenic stimuli (Shen et al., 2002). Consistent with the promotion of chondrocyte differentiation, STRA13 promotes neuronal but represses mesodermal and endodermal differentiation (Boudjelal et al., 1997), and SHARPs are abundantly expressed in a subset of mature neurons (Rossner et al., 1997). STRA13 deficient mice, although surviving to adulthood, develop autoimmune diseases accompanied by accumulation of spontaneously activated T and B cells (Sun et al., 2001). Furthermore, the mouse proteins are found to interact directly with clock Bmal1 protein and regulate the expression of biological clock regulator *Per* (Honma et al., 2002).

Another notable characteristic of DEC/STRA/SHARPs is that their expression is rapidly induced in response to various types of detrimental stimuli. In rats that undergo seizure induction by kainic acid, the levels of mRNA encoding SHARP1 or 2 are sharply elevated within 1 h (Rossner et al., 1997). Similarly, the expression of DEC1 is rapidly increased in response to hypoxia, a condition that closely mimics the microenvironment of tumors (Miyazaki et al., 2002; Ivanova et al., 2001). These findings link these transcription factors, particularly their elevated expression, to oncogenesis. Several lines of evidence support this possibility. First, expression of DEC1 appears to be deregulated in several tumor tissues. In paired samples from the colon, lung and kidney, DEC1 is abundantly expressed in the carcinomas but not in the adjacent normal tissues (Li et al., 2003). High levels of DEC1 mRNA are also detected in an array of cancer cell lines from a wide range of organs (Ivanova et al., 2001). Cells that lack the functional tumor suppressor VHL (von Hippel-Lindau) express higher levels of DEC1 (Ivanova et al., 2001). Second, DEC1 is antiapoptotic apparently through reducing caspase activities (Li et al., 2002). Forced expression of DEC1 effectively antagonizes apoptosis induced by serum starvation and causes a marked decrease on the activity of caspases-3/7 and 9 but not 8. In addition, a DEC1 mutant, lacking the DNA binding domain, shows neither antiapoptotic activity nor inhibitory effects on caspases (Li et al., 2002).

Caspases are cysteinyl aspartate-specific proteases and exist in normal cells as inactive forms, so called procaspases (Cryns and Yuan, 1998; Budihardjo et al., 1999). Upon apoptotic stimuli,

procaspases undergo proteolytic processing in a cascading activation manner, which results in the formation of active caspases. There are two major activation pathways: the cell surface death receptor pathway and the mitochondrial pathway (Budihardjo et al., 1999). For example, binding of FasL to its receptor results in the formation of the death-inducing signaling complex, which recruits and subsequently activates the upstream procaspase such as procaspase-8 (Ashkenazi and Dixit, 1999). In contrast, the mitochondrial pathway is initiated by intracellular death signals, leading to the formation of apoptotic protein complexes (Green and Reed, 1999). The complexes initiate the activation cascade from procaspase-9 to down-stream targets such as procaspase-3. Caspase activation, although following a sequential cascade, is regulated by several families of proteins. The inhibitor of apoptosis (IAP) family of proteins emerges as one of the major classes of proteins that negatively regulate apoptosis. Several IAP proteins are found to interact directly with and inhibit the activity of caspases (Yang and Li, 2000; Miller, 1999; LaCasse et al., 1998; Kasof and Gomes, 2001; Reed, 2001).

In this study, we report that DEC1 is a transcription activator of survivin, a member of the IAP family. In the paired tumor-normal tissues, survivin and DEC1 exhibited a paralleled expression pattern. In co-transfection assays, DEC1 activated a survivin promoter reporter but repressed a DEC2 promoter reporter. In contrast to the repression, the activation was delayed and depended on serum concentrations and cycle phase. Studies with deletion and substituted mutants located, in the proximal promoter, two Sp1 sites that supported the DEC1-mediated

activity. Both DEC1 and Sp1 were involved in the binding of these Sp1 sites. The reported activation of the survivin promoter provides a molecular explanation for the oncogenic involvement of DEC1 and represents the first example that DEC1 and other members in this class can act as transcription activators depending on the genomic context of a target gene.

MATERIALS AND METHODS

Chemicals and supplies

Hydroxyurea, mimosine, nocodazole, tetracycline, thymidine, and anti-FLAG antibody were purchased from Sigma. Propidium iodide was from BD Biosciences. Antibodies against procaspases and XIAP were from Cell Signaling. Antibody against Sp1 was purchased from Geneka Biotechnology. Antibodies against cIAP-1, cIAP-2 and survivin were from Santa Cruz. Goat anti-rabbit-IgG conjugated with alkaline phosphatase or horseradish peroxidase and ECL substrate were from Pierce. DMEM media and LipofectAMINE were from Invitrogen. Dual-Luciferase Reporter reagent and DNA binding buffer were from Promega. Unless otherwise indicated, all other reagents were purchased from Fisher Scientific.

Tissue collection and processing

Samples were collected from patients who underwent surgical resection for histologically confirmed adenocarcinoma. As paired controls, specimens from the adjacent, grossly normal tissues were harvested. The samples (3 or 4 pairs/each organ) were collected from the colon, kidney, and lung with four pairs from each organ. The age of the patients was between 23 and 68 with seven male and five female. The size of tumors was generally 2-5 cm in diameter, and the degree of differentiation of tumors was moderate or poor as determined by pathological examination. Samples were freshly processed for RNA isolation and protein extraction. Total RNA was isolated with a Tri-reagent as described previously (Xie et al., 2003). For the

preparation of protein extracts, tissues were homogenized in lysis buffer (20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.2 mM PMSF and 1 mM DTT). The homogenates were centrifuged at 12,000 g for 30 min to remove any insoluble precipitates. The protocol of using human pathological tissues was reviewed by the Institutional Review Board.

Reverse transcription coupled polymerase chain reaction (RT-PCR)

RT-PCR experiments were performed with a ThermoScript I kit. Total RNA (2 µg) was subjected to the synthesis of the first strand cDNA with an oligo(dT) primer and a ThermoScript reverse transcriptase. The reactions were incubated initially at 50°C for 30 min, and then at 60°C for 60 min after additional reverse transcriptase was added. The cDNAs were then subjected to PCR amplification with cycling parameters as follows: 95°C for 30s, 52°C for 30s and 68°C for 30 or 40s for a total of 32 cycles. The primers for DEC1 amplification were: 5'-GTCTGTGAGTCACTCTTCAG-3', 5'-GAGTCTAGTTCTGTTTGAAGG-3'; the primers for survivin amplification were: 5'-TCAAGGACCACCGCATCTCTAC-3', 5'-GCACTTTC-TTCGAGTTTCC-3'; and the primers for β-actin amplification were: 5'-GTACCC-TGGCATTGCCGACAGGATG-3', 5'-CGCAACTAAGTCATAGTCCGCC-TA-3'. The PCR-amplified products were analyzed by agarose gel electrophoresis.

Plasmid

Expression constructs for DEC1 and mutants as well as the DEC2 promoter reporter

(pDEC2-luc) were described previously (Li et al., 2002; 2003). Survivin promoter reporters were prepared with the pGL3-basic luciferase vector (Promega). Deletion mutants of survivin reporters were generated by PCR with the pSurvivin-6270 serving as the template. Preparation and characterization of the pSurvivin-6270 reporter were described elsewhere (Li and Altieri, 1999). Substitution mutants (pSurvivin240-M and pSurvivin154-M) were also prepared with PCR with primers containing multiple nucleotide substitutions. The sequences of the primers to introduce substitutions are: 5'-CTACGCGTAATAAGGAACGAGCTGGTGATGTATCG-CTGGGTGCACCGCG-3' (for pSurvivin240-M), and 5'-CTACGCGTCCCGGCACACC-CCTAGTTATCAAGTTTCTACTCCAGAAGGC-3' (for pSurvivin154-M). All mutated constructs were subjected to sequencing analysis to confirm the desired mutation being made without secondary mutations.

Co-transfection experiment

Co-transfection experiments were performed with DEC1 stable transfected or the parent line (293T). The preparation and characterization of DEC1 stable transfectants were described previously (Li et al., 2002). Cells were plated in 24-well plates in DMEM media supplemented with 10% fetal bovine serum at a density of 1.6×10^5 cells per well. Transfection was conducted by lipofection with LipofectAMINE according to the manufacturer's instruction. Transfection mixtures contained DEC1 or a mutant construct (100 ng), reporter plasmid (100 ng) and the pRL-TK *Renilla* plasmid (1 ng). If 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 PBS and resuspended in passive lysis buffer (Promega). The lysed cells were subjected to 2 cycles of freezing/thawing. The reporter enzyme activities were assayed with a Dual-Luciferase Reporter Assay System. This system contained two substrates, which were used to determine the activity of two luciferases sequentially. The firefly luciferase activity, which represented the reporter gene activity, was initiated by mixing an aliquot of lysates (20 μ l) with Luciferase Assay Reagent II. Then the firefly luminescence was quenched and the *Renilla* luminescence 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 a case that 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.

Some co-transfection experiments were performed in media containing a specific cell cycle blocker. In addition to serum starvation, four commonly used blockers were included: hydroxyurea (3 mM), minosine (0.4 mM), thymidine (2 mM) and nocodazole (0.5 μ g/ml). These blockers are known to arrest cells at G1, late G1, S and G2/M, respectively. The blockers were added 3 h after the cells were transfected. The treated cells were cultured for additional 30 h. Some cells were lysed and analyzed for reporter activities, whereas others were harvested, ethanol-fixed, stained by propidium iodide and analyzed for cell cycles (BD FACSCaliber).

Electrophoretic mobility shift assay (EMSA)

DEC1 stable transfected cells (293T) were cultured in the presence or absence of tetracycline (1 µg/ml) for 36 h and nuclear extracts were prepared with a nuclear extraction kit (Active Motif). Nuclear proteins (10 µg) were incubated with radiolabeled double-stranded oligonucleotides (Table I) in a final volume of 10 µl containing 1X DNA binding buffer. For competition experiments, nuclear extracts were first incubated with excess cold probe (50x) and then mixed with the radiolabeled probe. Oligonucleotides with a disrupted Sp1 site were also used in the competition assays. For super-shift assays, an anti-DEC1 or anti-Sp1 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 (Xie et al., 2002). The anti-DEC1 antibody against the C-terminal peptide was described elsewhere (Li et al., 2002). Protein concentration was determined with BCA assay (Pierce) with bovine serum albumin as the standard. Data are presented as mean ± SD of at least four separate experiments, except where results of blots are shown in which case a representative experiment is depicted in the figures. Comparison between two values were made with Student's *t* test at $p < 0.05$.

RESULTS

DEC1 selectively increases the expression of survivin among IAP proteins

Overexpression of DEC1 results in decreased activities of caspases-3, 7 and 9, but not caspase 8.

To determine if DEC1 selectively represses the expression of procaspases, stable transfected lines inducibly expressing DEC1 or the DNA binding mutant (DEC1-M) were cultured in the presence or absence of tetracycline to modulate the expression of DEC1 and DEC1-M, and the levels of procaspases-3 and 8 were determined. As expected, addition of tetracycline caused a robust expression of DEC1 and DEC1-M (the mutant has a slightly higher electrophoretic mobility) (Figure 2-1A). In contrary to the hypothesis, however, neither DEC1 nor DEC1-M caused any changes on the levels of procaspase-3 or 8 (Figure 2-1A). Three clonal lines for each type of stable transfectants were tested, and consistent observations were made.

The inability of DEC1 to alter the expression of the procaspases suggests that DEC1 decreases the activity of the caspases through inhibiting the activation of procaspases. Several classes of proteins, notably the inhibitor of apoptosis (IAP) family (Yang and Li, 2000; Miller, 1999), have been shown to interfere with the processing and catalysis of several caspases. We next examined whether DEC1 up-regulates the expression of IAP proteins. DEC1 stable line underwent serum starvation for 36 h in the presence or absence of tetracycline, and cell lysates were collected and analyzed for the abundance of several major IAP proteins including cIAP-1, cIAP-2, XIAP, and survivin. Addition of tetracycline caused a robust induction of transfected

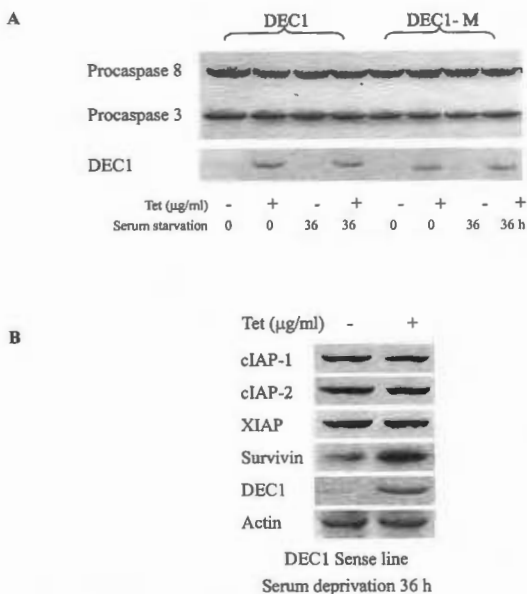


Figure 2-1. Expression of procaspases and inhibitor of apoptosis proteins in DEC1 stable transfected lines

(A) Abundance of and procaspases-3 and 8 in DEC1 and DEC1-M stable lines as a function of induced expression of DEC1. Stable transfected cells by DEC1 or DEC1-M (lacking the DNA binding domain) were seeded in 6-well plate. After reaching ~80% confluence, cells were treated with tetracycline (1 $\mu\text{g/ml}$) for 36 h. Cell lysates (5 μg) were subjected to SDS-PAGE. The immunoblot was incubated with an antibody against procaspases-3, 8 or DEC1. The primary antibody was then located by horseradish peroxidase-conjugated goat anti-rabbit IgG and visualized with chemiluminescent substrate. (B) Abundance of IAP proteins in DEC1 stable lines as a function of induced expression of DEC1. Stable transfected cells by DEC1 were seeded in 6-well plate. After reaching ~80% confluence, cells were treated with tetracycline (1 $\mu\text{g/ml}$) for 24 h. Cell lysates (5 μg) were subjected to SDS-PAGE. The immunoblot was incubated with an antibody against IAP proteins, DEC1 or β -actin. The blots were detected as describe above. Three clonal lines were used for each type of stable lines, and consistent results were obtained.

DEC1 (Figure 2-1B). However, only survivin was markedly increased in tetracycline-treated cells, suggesting that DEC1 is a selective regulator on the expression among IAP genes (Figure 2-1B).

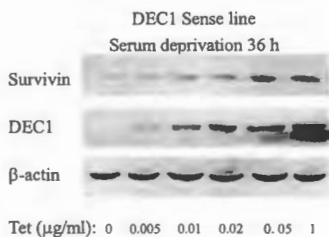
DEC1 and survivin show a paralleled expression pattern

In order to further establish the connection that DEC1 up-regulates the expression of survivin, two additional experiments were performed: survivin induction was monitored as a function of DEC1 levels, and expression patterns of DEC1 and survivin were determined among paired tumor-normal tissues from the kidney and lung. The DEC1 stable line was cultured in media containing tetracycline at various concentrations (0-1 μ g/ml) and the levels of DEC1 and survivin were monitored. Consistent with the notion that DEC1 up-regulates survivin, tetracycline-induced expression of DEC1 proportionally increased the levels of survivin (Figure 2-2A). In order to determine whether the correlative expression pattern occurs in vivo, samples from paired tumor-normal tissues were analyzed for the abundance of DEC1 and survivin. As shown in Figure 2-2B, both DEC1 and survivin were expressed markedly higher in the carcinomas than the adjacent normal tissues. Such a tumor-related increase was also detected by RT-PCR on both DEC1 and survivin (Figure 2-2C), suggesting that DEC1-mediated induction of survivin is achieved by increasing the levels of survivin mRNA. In summary, the paralleled expression pattern in tumor-normal samples and the inducible expression of survivin by DEC1 in the DEC1 stable line support the notion that DEC1 is an activator on the expression of survivin.

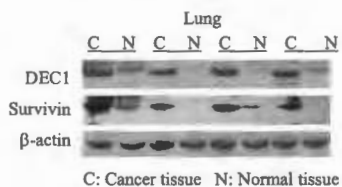
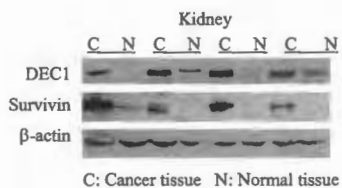
Figure 2-2. Induction of survivin by DEC1 and abundance of survivin in paired tumor and normal tissues from the kidney and lung

(A) Induction of survivin is proportionally correlated with the levels of DEC1 DEC1 stable cells were seeded in 6-well plate. After reaching ~80% confluence, cells were treated with tetracycline at various concentrations (0-1 µg/ml) for 36 h. Cell lysates (5 µg) were analyzed for the expression of survivin, DEC1 and β-actin. *(B) Paralleled expression of DEC1 and survivin in paired tumor and normal samples for the kidney and lung* Tissue homogenates (50 µg) from paired tumor-normal tissues were subjected to SDS-PAGE, and the immunoblot was incubated with the antibody against DEC1, survivin or β-actin. *(C) DEC1 and survivin mRNA in paired tumor-normal tissues for the kidney*: Total RNA (5 µg) of carcinoma-normal paired samples from the colon, kidney and lung was subjected to RT-PCR analyses with a ThermoScript I kit. For PCR amplification, a master tube containing all common reagents was prepared and equally distributed to individual PCR reaction tubes (DEC1, survivin and β-actin). PCR amplification was conducted with cycling parameters as follows: 95°C for 30s, 52°C for 30s and 68°C for 30 or 40s for a total of 32 cycles. The PCR-amplified products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

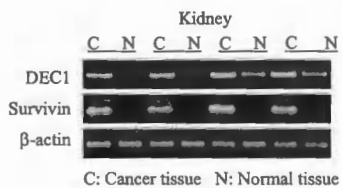
A



B



C



DEC1-mediated induction of survivin varies depending on serum concentrations

Solid tumors are poorly vascularized, thus hypoxic and deficient in nutrients (Vaupel et al., 2001). It is likely that the levels of DEC1, serum concentrations and oxygen tension collectively determine the overall induction of survivin. We next examined the effects of serum concentrations on the DEC1 mediated induction of survivin. As shown in Figure 2-3A, the expression of DEC1 is markedly induced by tetracycline independently of serum concentrations. In contrast, the levels of survivin varied markedly depending on the concentrations of serum in media. Generally, higher serum concentrations supported higher expression of survivin, and in a given serum concentration, higher levels of DEC1 supported higher expression of survivin. Cells cultured with lower serum concentrations (0-2%) exhibited markedly higher induction of survivin (between the presence and absence of tetracycline in a given serum concentration) (Figure 2-3A). Remarkably, the induction of survivin in cells cultured with less serum, although profound, did not exceed the levels in cells cultured with higher serum concentrations (e.g., 5%) (Figure 2-3A).

Two mechanisms, namely increased survivin stability and/or expression, could be responsible for increased level of survivin. To distinguish these possibilities, we performed a time course study on the changes of the survivin levels in reference to the levels of β -actin. Both DEC1 and DEC1-M lines were included in this study. As shown in Figure 2-3B, little changes on the survivin levels were observed in the first 12 h-serum starvation. However, a rapid decline was detected after a 12 h-incubation (Figure 2-3B). Such time-dependent changes occurred in both

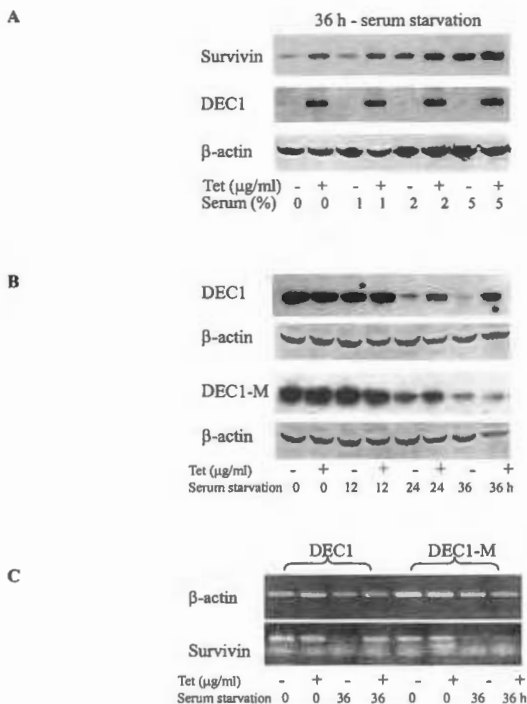


Figure 2-3. Time and serum-dependence of DEC1-mediated induction on survivin (A) Effects of serum concentrations on the induction of survivin DEC1 stable cells were seeded in 6-well plate. After reaching ~80% confluence, cells were cultured in media containing various amounts of serum (0-5%) in the presence or absence of tetracycline (1 µg/ml) for 36 h. Cell lysates (5 µg) were analyzed for the expression of survivin, DEC1 and β-actin. (B) *Time-course study on DEC1-mediated induction of survivin* DEC1 stable cells were cultured in the presence or absence of tetracycline (1 µg/ml) for 0-36 h. Cell lysates (5 µg) were analyzed for the expression of survivin, DEC1 and β-actin. (C) Abundance of survivin mRNA in DEC1 and DEC1-M lines DEC1 or DEC1-M stable lines were cultured in the presence or absence of tetracycline (1 µg/ml) for 36 h. Total RNA (5 µg) was subjected to RT-PCR analyses with a ThermoScript I kit for the levels of survivin and β-actin mRNA as described in the legend of Figure 2-2.

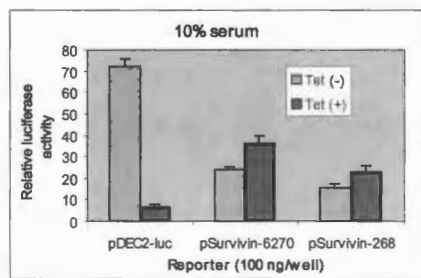
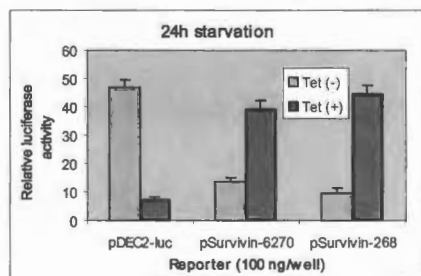
DEC1 and mutant lines, and independently of tetracycline. In contrast, in the 24 and 36 h-cultures, markedly higher levels of survivin were detected in the presence of tetracycline, but such increases were detected only in the DEC1 but not DEC-M line. Similarly, tetracycline-induced expression of DEC1 but not DEC1-M supported higher levels of survivin mRNA (Figure 2-3C). Addition of actinomycin D, an inhibitor on RNA synthesis, completely abolished DEC1-mediated increase on survivin mRNA (data not shown).

DEC1 stimulates the survivin promoter

The increase on survivin mRNA and the repressed increase by actinomycin D suggest that DEC1-mediated induction of survivin is achieved through transcription activation. We next determined whether DEC1 directly activates the survivin promoter. Three reporters were included in this study: pSurvivin-6270, pSurvivin-268, and pDEC2-luc. The rationale for the reporter selection was that these reporters contain various numbers of E-box elements and DEC1 has been shown to alter reporter activity through E-box DNA elements (Figure 2-4A) (Ivanova et al., 2001). The pSurvivin-268 reporter was derived from the 3' sequence of the pSurvivin-6270 reporter, and both starts from nucleotide -39 (Li and Altieri, 1999). More importantly, the pSurvivin-6270 reporter contains more than 30 E-box elements, whereas no E-box is present in the pSurvivin-268 reporter. The pDEC2-luc reporter contains a 1,888 bp-upstream sequence from the DEC2 gene, where two E-box elements (CACGTG) are present (Li et al., 2003). We have recently demonstrated that DEC1 represses the pDEC2-luc reporter

Figure 2-4. DEC1 activates the survivin promoter but represses the DEC2 promoter

(A) Diagrammatical presentation of pDEC2-luc, pSurvivin-6270 and -268. (B) Activation of the survivin reporters and repression of pDEC2-luc in full-media 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 re-transfected cells were cultured with media containing 10% serum in the presence or absence of tetracycline (1 µg/ml) for 24 h. The cells were collected, washed once with PBS 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. (C) Activation of the survivin reporters and repression of pDEC2-luc in serum free media 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 re-transfected cells were cultured with serum free media in the presence or absence of tetracycline (1 µg/ml) for 24 h. The reporter activities were determined as describe above.

A**B****C**

largely through the E-box in the proximal promoter region (49, 56). It should be emphasized that although the pSurvivin-6270 reporter contains numerous E-box elements, none of them is identical to the E-box in the pDEC2-luc reporter (Figure 2-4A).

Since DEC1-mediated induction of survivin was markedly more evident in media supplemented with lesser serum ($\leq 2\%$) (Figure 2-3A), the reporter assays were performed in full (10% serum) or serum free media. In the presence of serum, induction of DEC1 (addition of tetracycline) modestly stimulated the activity of both pSurvivin-6270 and 268 reporters (Figure 2-4B). In the absence of serum, however, both survivin reporters were markedly stimulated (Figure 2-4C). The pSurvivin-268 reporter exhibited a higher activation than the pSurvivin-6270 (Figure 2-4C). In a striking contrast, the pDEC2-luc reporter was repressed by more than 90%, whether or not serum was supplemented (Figure 2-4B and C).

Activation of pSurvivin-268 was delayed and cycle-dependent

The activation of the survivin reporter and repression of the pDEC2-luc reporter suggest that DEC1 regulates the transcription through more than one mechanism. A conceivable explanation for such opposing activities is that DEC1 is a part of a large transcription regulatory complex, and this complex rather than DEC1 alone determines the overall transcription activities (assumed that the complex varies among different target genes). Another possibility is that repression of pDEC2-luc is achieved through direct mechanism, whereas activation of

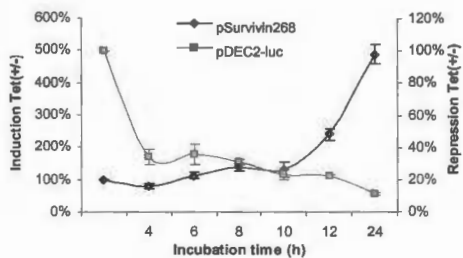
survivin reporters is through indirect mechanism or *vice versa*. It is expected that the direct mechanism would have an earlier onset than the indirect mechanism. In order to test this possibility, time-course studies were performed with pDEC2-luc and pSurvivin-268. As shown in Figure 2-5A, activation of the pSurvivin-268 reporter was not evident during the first 10 h-incubation, and the activation was increased with prolonged incubation, particularly after 10 h-incubation. In contrast, the pDEC2-luc reporter was markedly repressed as early as 4 h, and the overall repression was rather steady during the entire period of incubation (Figure 2-5A). The values from the 4 h-cultures in the absence of tetracycline were expressed as 100%.

The difference on the onset between repression and activation points to two important possibilities: (a) pDEC2-luc is a more sensitive target (requiring less DEC1) than pSurvivin-268; and (b) activation of pSurvivin-268 requires the synthesis of additional factors. Apparently, comprehensive experiments are required to definitively establish the involvement of each possibility. However, an effort was made to gain an initial insight by testing whether repression and activation are equally altered by cell cycle arrest. The rationale for this strategy is that the expression of survivin is regulated in a cycle-dependent manner (Li et al., 1998), and DEC1 mouse homologue STRA13 has been shown to delay cell cycle progression into S phase (Seimiya et al., 2002). DEC1 stable transfected cells were transfected again with pSurvivin-268 or pDEC2-luc and synchronized with cell cycle blockers. As shown in Figure 2-5B, DEC1 caused a marked repression of pDEC2-luc independently of cycle blockers. In

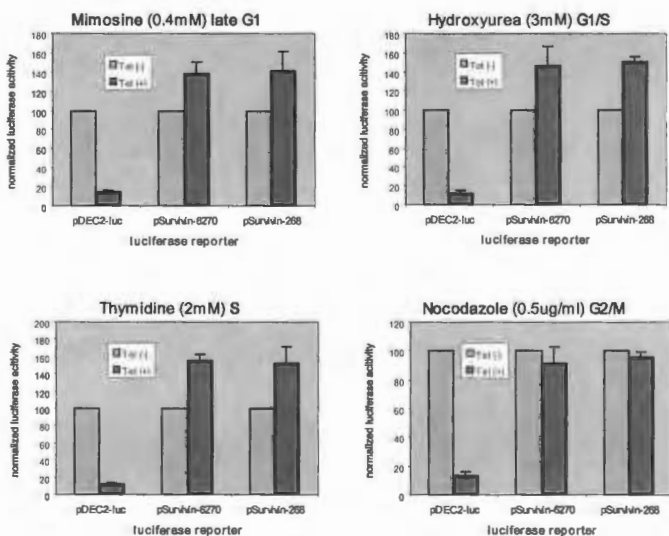
Figure 2-5. Activation of the survivin promoter by DEC1 is delayed and varies depending on cell cycle blockers

(A) *Activation of the pSurvivin-268 is delayed* 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 re-transfected cells were cultured with serum free media in the presence or absence of tetracycline (1 $\mu\text{g/ml}$) for 0-36 h. The reporter enzyme activities were determined as describe above. The values from the 4 h-cultures in the absence of tetracycline were expressed as 100%. (B) *Activation of pSurvivin-268 varies depending on cell cycle blockers* DEC1 stable transfected cells were cultured in 24-well plates to ~80% confluence and transfected again with a reporter construct (100 ng) and the pRL-TK *Renilla* (1 ng). The re-transfected cells were cultured in media containing no or a specific cycle blocker (3 mM hydroxyurea 3 mM, 0.4 mM mimosine, 2 mM thymidine and 0.5 $\mu\text{g/ml}$ nocodazole). The cells were cultured in media supplemented with 10% serum and in the presence or absence of tetracycline (1 $\mu\text{g/ml}$) for 24 h. The reporter enzyme activities were determined as describe above. The values from the cultures in the absence of tetracycline were expressed as 100%.

A



B



contrast, the activation of pSurvivin-268 varied depending on the blockers. A moderate activation was detected in cells treated with hydroxyurea, minosine and thymidine. In contrast, nocodazole, a cycle blocker arresting cells at G2/M, showed little effects on the DEC1-mediated activation on the reporter. It should be emphasized that hydroxyurea repressed but nocodazole activated the pSurvivin-268 reporter compared with vehicle controls (in the absence of DEC1), and treatment with a blocker generally achieved a 65-80% of cells arrested at a designated cycle as determined by DNA content analysis (data not shown).

Requirements of functional domains in DEC1 to activate the survivin transcription

DEC1 has several functional domains, and some of them (e.g., the orange domain) likely interact with other proteins. To determine which functional domains are required for the activation of survivin reporter, several DEC1 mutants with certain functional domains selectively disrupted were prepared and tested for the ability to stimulate the pSurvivin-268 reporter. These mutants are diagrammatically shown in (Figure 2-6, Left). The results of the reporter assays with the DEC1 mutants on pSurvivin-268 were summarized in Figure 2-6 (Right). Deletion mutants retaining DNA binding ability (DEC1₁₋₃₄₇, DEC1₁₋₂₇₀, DEC1₁₋₁₉₇ and DEC1₁₋₁₅₀) all exhibited some transactivation activities depending on the length of a mutant. For example, DEC1₁₋₃₄₇ exhibited ~60% of the activity of DEC1, whereas DEC1₁₋₁₅₀, with a longer C-terminal deletion, had only ~20% of the activity of DEC1 (the activity from the empty

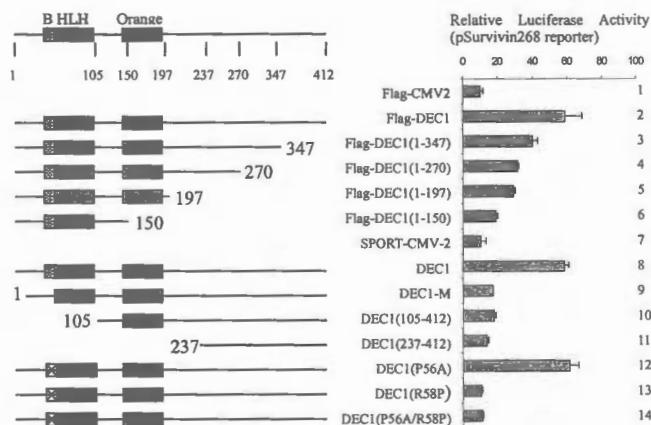


Figure 2-6. Requirements of functional domains in DEC1 to activate the pSurvivin-268
Activation of the pSurvivin-268 Cells (293T) were cultured in 24-well plates and transiently transfected with DEC1 or a mutant (100 ng), pSurvivin-268 (100 ng) and the pRL-TK *Renilla* (1 ng). The transfected cells were cultured in serum free media for 24 h, and lysates were collected and analyzed for luciferase activities. Similarly firefly luminescence signal was normalized based on the *Renilla* luminescence signal.

vector was subtracted). In contrast, the mutants with the DNA binding domain or an N-terminal deletion (DEC1-M, DEC1₁₀₅₋₄₁₂, and DEC1₂₃₇₋₄₁₂) showed little activity toward the pSurvivin-268 reporter. These mutants were previously shown to have no DNA binding activity (Ivanova et al., 2001). Among the substitution mutants, the DEC1_{P56A} mutant showed a higher activity than DEC1 in activating the pSurvivin-268 reporter, whereas the other two mutants (DEC1_{R58P} and DEC1_{P56A_R58P}) showed no activation activity. DEC1_{P56A} but not DEC1_{R58P} nor DEC1_{P56A_R58P} was shown to bind to E-box. It should be emphasized that Western analyses detected comparable expression with all constructs (results not shown).

Both DEC1 and Sp1 bind to the proximal promoter of survivin

Motif analyses identified a clustered Sp1/CDE region (cycle dependent element) in the proximal region of survivin promoter and has been shown to mediate its basal expression (Li and Altieri, 1999). Some of the Sp1 sites are slightly altered and arranged in a modified configuration (Figure 2-7A). In order to identify DNA sequences that act as potential binding sites for DEC1, ten oligonucleotides were synthesized to span the entire region with partial sequences overlapped on one or both ends of an oligonucleotide (Table 1). Equal amounts of extracts from control or tetracycline-treated cells were allowed to bind to the double stranded oligonucleotides individually, and shifted bands were detected by non-denaturing PAGE. As shown in Figure 2-7B, one or more shifted bands were detected with seven out of ten oligonucleotides including oligonucleotides 41-68, 55-84, 85-114, 105-126, 115-144, 123-158,

Table 1. Sequences of Oligonucleotides for Electrophoretic Mobility Shift Assay

Oligonucleotide	Sequence
41-68	5'-CCCCGCGGC GCGCCATTAACCGCCAGAT-3'
55-84	5'-GTGCGTCCCGACATGCCCGCGGCGCGCC-3'
76-105	5'-GGGGTGGAC <u>CGCCT</u> AAGAGGGCGTGCGCTCC-3'
85-114	5'-GGCCGCGGGGGTGGAC <u>CGCCT</u> AAGAGGGC-3'
105-126	5'-CTACTCCAGAAAGGCCGCGGGG-3'
115-144	5'-CCCCGCGCCGCCCGCCTCTACTCCAGAA-3'
123-158	5'-CAACTCCCGGCACACCCCGCGCCGCCCGCCTCTAC-3'
145-174	5'-CGCGGGGGAGGACTACAACCTCCCGGCACA-3'
175-204	5'-CTGGGTGCACCGCGACCAC <u>GGCAG</u> AGCCA-3'
205-234	5'-TGT <u>GGCAG</u> GGACGAGCTGGCGCGGCGTCG-3'

Note: Oligonucleotides are numbered based on the genomic sequence described previously (27), and the sequences underlined are Sp1 canonical or Sp1 sites.

Figure 2-7. DEC1 selectively increases DNA binding among Sp1 sites in the proximal promoter of survivin

(A) Diagrammatical presentation of the location of Sp1 sites (B) Expression of DEC1 selectively alters DNA binding to Sp1 sites 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 in a final volume of 10 µl containing 1X DNA binding buffer. For competition experiments, nuclear extracts were first incubated with excess cold probe (50 X in lane 1 or 10 X lane 3) and then mixed with the radiolabeled probe. The protein-DNA complexes were resolved in 6% polyacrylamide gel electrophoresis and visualized by autoradiography. *(C) Disruption of shift bands by anti-DEC1 or Sp1 antibody* The gel mobility shift assays were performed as described above but in the presence of an antibody against DEC1, Sp1 or Flag tag. The protein-DNA complexes were resolved in 6% polyacrylamide gel electrophoresis and visualized by autoradiography.

A

Sp1-226

268 - CGCGTTCTTTGAAAGCAGTCGAGGGGGCGCTAGGT - 234 - GT GGGCAG GGACGAGCTGGCGCG

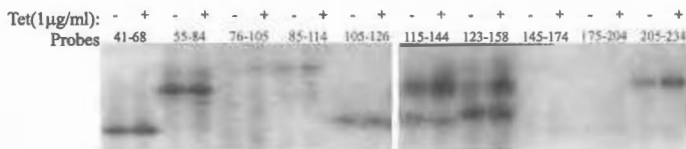
G - 209 - CGTCGCTGGGTGCACCGGACCACGGCAGAGCCACGCGGCGGGAGGACTACAAC - 154 - T

Sp1-127

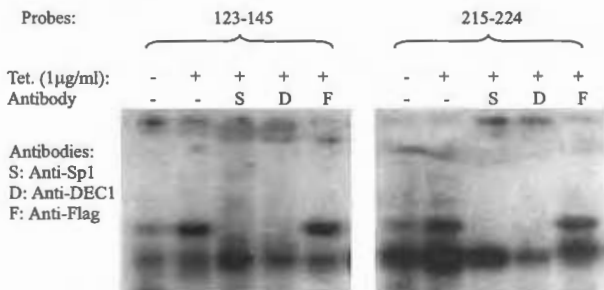
CCCGGCACACCCCGCCCGCCCGCCT - 125 - TACTCCAGAAAGCCGCGGGGGTGACCGCCTAA

GAGGGCGTGCGCTCCCGACATGCCCGCGGGGGCCATTAAACCGCCAGATTT - 39

B



C



205-234. More importantly, induction of DEC1 differentially affected the binding of the oligonucleotides. Markedly higher binding activities were detected with oligonucleotides 115-144, 123-158 and 204-234 with cells cultured in the presence of tetracycline. In addition, two shifted bands were detected with oligonucleotides 115-144 and 123-158, but only the upper shifted band was intensified as a result of induced expression of DEC1, suggesting that the detected binding was highly specific. It should be emphasized that the binding specificity with all oligonucleotides was also established with competition assays in the presence of corresponding unlabeled probes (data not shown).

Oligonucleotides 115-144 and 123-158 share an Sp1 complex site (SP1-127), whereas oligonucleotide 204-234 contains a typical Sp1 element (Figure 2-7A), indicating that the shifted bands with these oligonucleotides represent DNA-Sp1 complexes. In order to directly test this possibility, EMSA experiments were performed with an anti-Sp1 antibody. As shown in Figure 2-7C, addition of the anti-Sp1 antibody caused disappearance of the shifted band with oligonucleotide 205-234 as well as the upper shifted band with oligonucleotide 123-158. A super-shifted band with a markedly lesser intensity was also detected with each oligonucleotide. Interestingly, a rather high background (so-called smeared bands) was observed between the original- and super-shifted bands, suggesting that many intermediated species of DNA-protein complexes are formed as a result of the addition of the anti-Sp1 antibody. Given the fact that binding to these oligonucleotides was increased by the presence of DEC1, we next examined

whether DEC1 is part of the DNA-protein complexes. Similarly, an antibody against DEC1 was added to binding mixture. As observed with the anti-Sp1 antibody, addition of the anti-DEC1 antibody resulted in the disappearance of the originally shifted bands accompanied by super-shifted bands with a markedly lesser intensity (Figure 2-7C). Addition of an anti-FLAG antibody, however, caused no changes on the relative intensity of the shifted bands, further establishing the specificity of the observed bindings.

Sp1 sites-127 and 226 additively activate the survivin promoter in response to DEC1

In order to determine whether binding to Sp1 site-127 or 226 is responsible for the activation of the survivin reporter, deletion and substitution mutants were prepared and tested for the responsiveness to DEC1. Similarly, the DEC1 stable line was transfected again with a reporter mutant and cultured in the presence or absence to modulate the expression of DEC1. As shown in Figure 2-8, deletion or mutagenic disruption of the Sp1 site-226 decreased the responsiveness to DEC1 by as much as 30%. An additional 40% decrease was detected with the mutant that had Sp1 site-127 also disrupted in addition to Sp1 site-226 (pSurvivin-154M). Therefore, binding to these two Sp1 sites is responsible for as much as 70% of the overall activation mediated by DEC1 (Figure 2-8).

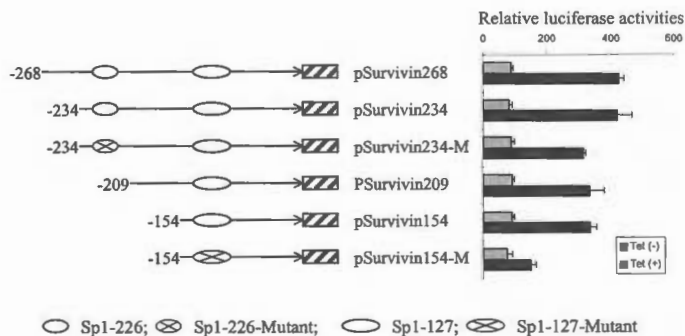


Figure 2-8. Sp1 sites 127 and 226 support DEC1-mediated activation of survivin DEC1 stable transfected cells were cultured in 24-well plates at ~80% confluence and transfected again with a reporter mutant (100 ng) and the pRL-TK *Renilla* (1 ng). The re-transfected cells were cultured with serum free media in the presence or absence of tetracycline (1 μ g/ml) for 24 h. The reporter enzyme activities were determined as describe above. The values were normalized based on the reading on pRL-TK *Renilla* luciferase

DISCUSSION

Recent reports from this and other laboratories have linked deregulated expression of DEC1 to oncogenic process (Li et al., 2002; 2003; Ivanova et al., 2001). DEC1 is abundantly expressed in the tumor but not in the adjacent normal tissues. Tumorigenic environment such as hypoxia markedly induces the expression of DEC1 and the induction is abolished by von Hippel-Lindau tumor-suppressor protein (Ivanova et al., 2001). In stable transfected cells, forced expression of DEC1 protects against apoptosis, and the overall antiapoptotic activity is well correlated with decreased activity of several major caspases. In this study, we report a molecular mechanism for the decrease on the caspase activity. DEC1 transcriptionally upregulates the expression of survivin, an antiapoptotic protein that interferes with the processing and catalysis of caspases. DEC1 and survivin exhibit a paralleled expression pattern in paired tumor-normal tissues. In co-transfection experiments, DEC1 stimulates the survivin promoter, and this mechanism relies on the physical interactions with Sp1 sites in the proximal promoter. These findings collectively establish that the survivin gene is a transcription target of DEC1.

The DEC1-directed regulation likely represents an important mechanism that governs the transcription of the survivin gene. Several lines of evidence support this notion. First, both DEC1 and survivin are abundantly expressed in the carcinomas but not the adjacent normal tissues (Figure 2-2B), providing cellular localization that supports for the regulator and target relationship (DEC1 and survivin). Second, DEC1 and survivin share an overlapping

cycle-dependent expression. The expression of survivin is regulated in a cycle-dependent manner with a robust expression in the G2/M phase of the cell cycle (Li et al., 1998). Consistent with the cycle-related expression, cycle blockers hydroxyurea (S phase) and nocodazole (G2/M phase) markedly induce DEC1 (Li et al., 2002), suggesting that increased expression of DEC1 is an initial event leading to the expression of survivin. Third, forced expression of DEC1 alters the cycle-dependent expression of survivin. Cells synchronized by G1 blocker mimosine express undetectable survivin (Li et al., 1998), however, transfection of DEC1 in mimosine-synchronized cells causes a modest activation of the survivin promoter. As a matter of fact, forced expression of DEC1 activates the survivin promoter independently of the cycle phase (Figure 2-4 and 5) with an exception of nocodazole.

The inability of transfected DEC1 in nocodazole-synchronized cells causes additional activation suggests that DEC1 and this cycle blocker act redundantly on the induction of survivin. However, there are several important differences on the *cis*-DNA elements required for nocodazole- and DEC-mediated activation. In this study, Sp1 sites-127 and 226 in the proximal promoter of survivin are found to contribute as much as 70% to the activation of DEC1 (Figure 2-8). In contrast, nocodazole-mediated activation requires a 40 bp-proximal sequence containing two cycle dependent elements (CDE) and one cycle homology region (CHR) (Li and Altieri, 1999). Mutagenic disruption of one or more of these elements abolishes the ability to respond to nocodazole. It remains to be determined whether the CDE/CHR region

affects DEC1-mediated activation, or the Sp1 sites-127 and 226 affect nocodazole-mediated activity. It should be emphasized that other Sp1 sites (-151 and -171) in the survivin promoter have been shown to support constitutive activation of survivin in HeLa cells (Li and Altieri, 1999). However, no DNA binding to these sites is detected with 293T nuclear extracts (Figure 2-7B, oligonucleotides 145-174 and 175-204), suggesting that these sites represent cell type-specific regulation.

Binding to Sp1 sites-127 and 226 may not be the only event in DEC1-mediated activation on the survivin promoter. In DEC1 stable transfectants, marked induction of survivin is observed only in cells cultured at relatively low serum concentrations (Figure 2-3A), suggesting that the presence of DEC1 alone is not sufficient to effectively upregulate the expression of survivin. Similarly, less serum supports higher activation of the survivin promoter reporters (Figure 2-4B and C). Even with serum free media, the activation of the survivin reporter is very minimal during the first 10 h-incubation, and the activation continues to increase with prolonged incubation (Figure 2-5A). In contrast, the pDEC2-luc reporter is repressed by 60% as early as 4 h. The delayed onset on the activation of the survivin reporter suggests that DEC1 initiates more than one event (e.g., binding to Sp1 sites-127 and 226), and these events collectively determine the onset and magnitude of the activation.

Given the fact that the DEC1-mediated activation of the survivin reporter is largely achieved

through Sp1 sites-127 and 226, it is conceivable that increased expression of Sp1 is one of the events responsible for the delayed onset on the activation. It remains to be determined whether DEC1 actually upregulates the expression of Sp1, however, EMSA experiments have detected marked increases on Sp1 binding activity as a result of the induced expression of DEC1 (Figure 2-7B). In contrary to this possibility, increased Sp1 binding occurs only with Sp1 sites-127 and 226 but not with five others present in this region, although some of the other Sp1 sites are found to bind to Sp1 and related proteins (Li and Altieri, 1999; Figure 2-7B). Alternatively, Sp1 interacts with other proteins and the resultant complexes collectively determine DNA binding selectivity. In support of this possibility, mSHARP, a DEC1-related transcription factor, has been shown to directly interact with Sp1 (Azmi et al., 2003). In this study, we have demonstrated that addition of an anti-DEC1 antibody completely disrupt the shifted bands with Sp1 sites-127 and 227 (Figure 2-7C), suggesting that DEC1 is part of the Sp1-DNA complex. The disrupted binding instead of being super-shifted by the anti-DEC1 or even anti-Sp1 antibody suggests that DEC1-Sp1 complex presents a rigid steric conformation with a high selectivity toward a *cis*-DNA element. Post-translational modifications of Sp1 have been shown to alter Sp1-mediated DNA binding (Black et al., 2001; 1999; Rohlf et al., 1997; Su et al., 1999). However, it remains to be determined whether post-translational modifications of Sp1 are involved in the interaction with DEC1 or mSHARP.

It is interesting to notice that interactions between Sp1 and DEC1 result in transcription

activation, whereas Sp1-mSHARP complex represses the promoter of STRA13, the mouse homologue of DEC1 (Fujimoto et al., 2001). DEC1 and mSHARPI are highly identical (97%) in the bHLH region, but rather diverse (~35%) in other regions, particularly in the C-terminal half (Fujimoto et al., 2001). The high degree of sequence identity in the bHLH region suggests that this motif mediates direct interactions with Sp1. However, it remains to be determined whether the regions with relatively diverse sequences are responsible for the opposing activities on these two reporters. Apparently, the difference between the target genes (survivin *versus* STRA13) is another possibility that is likely responsible for the observed opposing activities. Consistent with this notion, Sp1, which is generally considered as a transcription activator, has been increasingly reported to exert transcription repression depending on the target genes, the types of cells and even the cycle phase (Black et al., 2001).

The DEC1-mediated activation of the survivin has important pathological significance. Survivin has been shown to antagonize apoptosis and promote cell division (Li et al., 1999; 2003). The expression of survivin is cell cycle-regulated with a marked increase in the G2/M phase (Li et al., 1998). The dual function (antiapoptosis-cell division), along with its cycle-dependent expression, provides an effective mechanism to ensure success of cell division. In this study, forced expression of DEC1 causes increased the activation of the survivin promoter independently of the cycle phase (with an exception of nocodazole, see discussed above). The DEC1-mediated alteration on the cycle dependent expression of survivin provides

a mechanism that minimizes cell death during oncogenic process regardless of a cycle phase (assumed that the deregulated expression of DEC1 in tumors occurs in a cycle-independent manner). Given the fact that DEC1 has a broad tissue distribution (Shen et al., 1997), DEC1-mediated activation may have physiological significance in cell proliferation and differentiation as well. In support of this possibility, STRA13-deficient mice develop autoimmune disease featured by accumulation of spontaneously activated T and B cells (Sun et al., 2001). Although these mice are generally normal, but the FasL surface expression is markedly suppressed. Dysfunctional Fas-FasL system results in decreased apoptosis of premature lymphocytes, leading to the development of autoimmune disease. The decreased expression of FasL in STRA13-deficient mice suggests that STRA13, like its human homologue DEC1 on the survivin, acts as a transcriptional activator. It remains to be determined whether Star13-mediated upregulation of FasL is achieved by STRA13-Sp1 complex. Interestingly, Sp1-dependent activation is a major pathway to support the expression of FasL (Kavurma et al., 2002).

DEC1-mediated activation, compared with repression, has several important differences. First, activation and repression differ on the time of onset. Repression of pDEC2-luc is profound as early as 4 h after transcription, whereas activation of pSurvivin-268 is not evident after 10 h-incubation, suggesting that activation requires one or more additional events (e.g., protein synthesis) (Figure 2-5A). Second, repression of pDEC2-luc is independent on serum

concentrations and cycle blockers, whereas activation of pSurvivin-268 is altered by these factors, suggesting that DEC1 is essential but not sufficient to effectively activate this reporter. Third, Sp1-sites support the activation of pSurvivin-268, whereas the E-box in the proximal region supports the repression of pDEC2-luc (Li et al., 2003), suggesting that direct DNA binding alone is sufficient to exert repression, whereas protein-protein interaction (e.g., DEC1-Sp1) is required to support activation (although DNA binding is also involved as discussed below). And finally, the C-terminal 65 residues are required for maximum activation but not repression (Li et al., 2003). It is intriguing that intact DNA binding domain in DEC1 is essential to transcription activation, although interaction with Sp1 is an identified mechanism. A conceivable explanation is that DNA binding mechanism and interactions with Sp1 are both involved in the activation of survivin. The DNA binding mechanism directly represses or activates a target gene, which, in cooperation with the DEC1-Sp1 complex, leads to the activation of the survivin promoter. The DNA binding-dependent transcription is likely responsible for the delayed onset on the activation.

In summary, our work points to several important conclusions. First, DEC1 antagonizes apoptosis accompanied by decreased activity of caspases, and in this study, we reports that DEC1 transcriptionally upregulates the expression of the survivin, providing a molecular explanation for its antiapoptotic activity. Second, the activation of the survivin is delayed and varies depending on serum concentration and cycle blockers, suggesting that DEC1-mediated

activation requires more than one event and operates in cell type and cycle-related manner. Third, DEC1 activates the survivin promoter, but represses the DEC2 promoter, providing dual transcription functionality mediated by DEC1. Given the fact that DEC1 has been implicated in oncogenic process (deregulated) and is generally considered to be transcriptionally repressive, the established activation of the survivin promoter provides a molecular explanation for its oncogenic involvement and represents the first example that DEC1 and other members in this class can act as transcription activators depending on the genomic context of a target gene.

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MANUSCRIPT III

DEC1 NEGATIVELY REGULATES THE EXPRESSION OF DEC2

THROUGH BINDING TO THE E-BOX IN THE PROXIMAL PROMOTER¹

ABSTRACT

Human DEC, mouse STRA and rat SHARP proteins constitute a new and structurally distinct class of the basic helix-loop-helix (bHLH) proteins. In each species, two members are identified with a sequence identity of >90% in the bHLH 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 proportional 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.

INTRODUCTION

The basic helix-loop-helix (bHLH) proteins are intimately associated with developmental events such as cell differentiation and lineage commitment (Littlewood and Evan, 1998; Bissonnette et al, 1994; Cromiller et al 1998; Nikoloff et al, 1992; Hirose et al, 1996; Massari and Murre 2000). The HLH domain in the bHLH motif is responsible for dimerization, whereas the basic region mediates DNA binding (Littlewood and Evan, 1998). Based on sequence alignment and domain analysis, human DEC, mouse STRA and rat SHARP constitute a new and structurally distinct class of bHLH proteins (Boudjelal et al, 1997; Fujimoto et al, 2001; Rossner et al, 1997; Shen et al, 1997). These proteins are distantly 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 (Littlewood and Evan, 1998; Dawson et al, 1995; Kokubo et al, 1999). 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 N-terminus (Littlewood and Evan, 1998; Fujimoto et al, 2001). Another major structural difference on the functional domains is that DEC/STRA/SHARPs, unlike Hairy/E(Spl)/Hes proteins, lack the C-terminal WRPW tetrapeptide motif (Fisher et al, 1996). Through this sequence, Hairy/E(spl)/Hes recruit corepressor Groucho to the transcription regulatory complex (Fisher et al, 1996). Recruitment of Groucho is responsible for a vast array of biological activities of Hairy/E(spl)/Hes proteins including cellular differentiation and lineage commitment (Giebel and Campos-ortega, 1997; Poortinga et al, 1998; Chen et al, 1999;

Hojo et al, 2000; Ohtsuka et al, 2001).

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 (Fujimoto et al, 2001). They exhibit an overlapping tissue distribution and their expression is highly elevated in response to environmental stimuli (Boudjelal et al, 1997; Fujimoto et al, 2001; Rossner et al, 1997; Shen et al, 1997). 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 (Rossner et al, 1997). In cultured human cells, both DEC1 and DEC2 are markedly induced in response to hypoxia (Miyazaki et al, 2002). 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-1 α and β , providing a molecular explanation on the co-regulatory phenomena of DEC1 and DEC2 during hypoxia response (Miyazaki et al, 2002). 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 (Boudjelal et al, 1997; Shen et al, 1997; Shen et al, 2002), maturation of lymphocytes (Sun et al, 2001) and regulation of molecular clock (Honma

et al, 2002). In a cell culture system, mouse STRA13 promotes neuronal but represses mesodermal and endodermal differentiation (Boudjelal et al, 1997). Consistent with the inductive effect on neuronal differentiation, rat SHARP proteins are abundantly expressed in a subset of mature neurons (Rossner et al, 1997). DEC1 has recently been shown to promote chondrocytes differentiation at the early and terminal stages (Shen et al, 2002). STRA13 deficient mice, although surviving to adulthood, develop autoimmune diseases accompanied by accumulation of spontaneously activated T and B cells (Sun et al, 2001). In addition, the mouse proteins are recently found to regulate the expression of biological clock regulator *Per* (Honma et al, 2002). In addition, 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 (Li et al, 2002). High levels of DEC1 transcript are also detected in an array of cancer cell lines derived from a wide range of organs (Ivanova et al, 2001). Cells that lack the functional tumor suppressor VHL (von Hippel-Lindau) express higher levels of DEC1 (Ivanova et al, 2001). Forced expression of DEC1 antagonizes serum deprivation-induced apoptosis and selectively inhibits the activation of procaspases (Li et al, 2002). These findings suggest that overexpression of DEC1 provides cells with unusual survival mechanism, 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 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, which is largely achieved through direct DNA binding to the E-box in the proximal promoter of DEC2.

MATERIALS AND METHODS

Chemicals and supplies

Tri-reagent, Flag-CMV vector and anti-Flag antibody were purchased from Sigma. The goat anti-rabbit-IgG conjugated with alkaline phosphatase or horseradish peroxidase and ECL substrate were from Pierce. DMEM media, LipofectAMINE and ThermoScript I RT-PCR kit were from Invitrogen. Dual-Luciferase Reporter Assay System and DNA binding buffer were from Promega. Unless otherwise indicated, all other reagents were purchased from Fisher Scientific.

Tissue collection and processing

Samples were collected from patients who underwent surgical resection for histologically confirmed adenocarcinoma. As paired controls, specimens from the adjacent, grossly normal tissues were harvested. The samples (12 pairs) were collected from the colon, kidney, and lung with four pairs from each organ. The age of the patients was between 23 and 68 with seven male and five female. The size of tumors was generally 2-5 cm in diameter, and the degree of differentiation of tumors was moderate or poor as determined by pathological examination. Samples were freshly processed for RNA isolation and protein extraction. Total RNA was isolated with a Tri-reagent as described previously (Zhang et al, 1999). For the preparation of protein extracts, tissues were homogenized in lysis buffer (20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.2 mM PMSF and 1 mM DTT). The

homogenates were centrifuged at 12,000 g for 30 min to remove any insoluble precipitates. The protocol of using human pathological tissues was reviewed by the Institutional Review Board.

Reverse transcription coupled polymerase chain reaction (RT-PCR)

The expression of DEC1 and DEC2 in human tissues and cultured cells was primarily determined by RT-PCR experiments with a ThermoScript I kit. Total RNA (2 µg) was subjected to the synthesis of the first strand cDNA with an oligo(dT) primer and a ThermoScript reverse transcriptase. The reactions were incubated initially at 50°C for 30 min, and then at 60°C for 60 min after additional reverse transcriptase was added. The cDNAs were then subjected to PCR amplification with cycling parameters as follows: 95°C for 30s, 52°C for 30s and 68°C for 30 or 40s for a total of 32 cycles. The primers for DEC1 amplification were: 5'-GTCTGTGAGTCACTCTTCAG-3', 5'-GAGTCTAGTCTGTGTTGAAGG-3'; the primers for DEC2 amplification were: 5'-CGCCATTAGTCCGACTTGGAT-3', 5'-TGGTTGAT-CAGCTGGACACAC-3'; and the primers for β-actin amplification were: 5'-GTACCCTGG-CATTGCCGACAGGATG-3', 5'-CGCAACTAAGTCATAGTCCGCCTA-3'. The PCR-amplified products were analyzed by agarose gel electrophoresis.

Plasmid

A cDNA encoding the full-length DEC1 was isolated by a cDNA-trapping method (Li et al, 2002; Hu and Yan, 1999). Several DEC1 mutant constructs were prepared by PCR with the

full-length DEC1 as the template. These mutants had a specific sequence deleted or one or more amino acids substituted. Some of the mutant constructs were prepared with the SPORT vector (the N-terminal truncated mutants), whereas others (the C-terminal truncated mutants) were prepared with the Flag-vector to facilitate immuno-detection. In some cases, a Kozak sequence was introduced for effective translation initiation. The DEC2 promoter reporter was prepared with the pGL3-basic luciferase vector (Promega). Human genomic DNA was isolated from the placenta with a DNA extraction kit (Qiagen) according to the manufacturer's instruction. A genomic fragment (-1,888 to +11) was generated by PCR with 5'-AACAGATG-AACTGAACGGACCG-3' and 5'-CCTCAGTGCAGTGTGAAAGTG-3'. This PCR fragment was ligated to the pGL3 vector. Deletion mutants of this reporter were prepared by endonuclease digestion followed by ligation or PCR.

Site-directed mutagenesis

The DEC2 promoter reporter had two E-box motifs that likely 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 QuickChange site-directed mutagenesis kit (Stratagene). Complementary oligonucleotides (5'-GATGGTACGTTCCGAACGGGAGCTGGGTGCTGG-3') were synthesized to target this region. To perform the substitutions, the primers were annealed to a

DEC2 promoter reporter and subjected to a thermocycler for a total of 15 cycles. The resultant PCR-amplified constructs were then digested with Dpn I to remove the non-mutated 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'-GAGACCTACAAATT**GGCGC**-AC**CCGCTC**ATCGAGAAAAGAG-3' with the nucleotides in bold 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 DMEM media supplemented with 10% fetal calf serum at a density of 1.6×10^5 cells per well. Transfection was conducted by lipofection with LipofectAMINE according to the manufacturer's instruction. Transfection mixtures contained DEC1 or a mutant construct (100 ng), reporter plasmid (100 ng) and the pRL-TK *Renilla* plasmid (1 ng). If 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 PBS and resuspended in passive lysis buffer (Promega). The lysed cells were subjected to 2 cycles of freezing/thawing. The reporter enzyme activities were assayed with a Dual-Luciferase Reporter Assay System. This system contained two substrates, which were

used to determine the activity of two luciferases sequentially. The firefly luciferase activity, which represented the reporter gene activity, was initiated by mixing an aliquot of lysates (20 μ l) with Luciferase Assay Reagent II. Then the firefly luminescence was quenched and the *Renilla* luminescence 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 a case that 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.

Electrophoretic mobility shift assay (EMSA)

Cells (293T) were transfected with DEC1 or a mutant and nuclear extracts were prepared with a nuclear extraction 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 μ g) were incubated with radiolabeled double-stranded oligonucleotides (5'-CGTTCCGCACGTGAGCTGGG-3') in a final volume of 10 μ l containing 1X DNA binding buffer. For competition experiments, nuclear extracts were first incubated with excess cold probe (50 or 10 X) and then mixed with the radiolabeled probe. Oligonucleotides with a disrupted E-box were also used in the competition assays. For super-shift 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.

Western analyses were conducted as described previously (Zhu et al, 2000). The anti-DEC1 antibody against the C-terminal peptide was described elsewhere (Li et al, 2002). Protein concentration was determined with BCA assay (Pierce) with bovine serum albumin as the standard. Data are presented as mean \pm SD of at least four separate experiments, except where results of blots are shown in which case a representative experiment is depicted in the figures.

RESULTS

DEC1 and DEC2 are inversely expressed in paired carcinomas and adjacent normal tissues

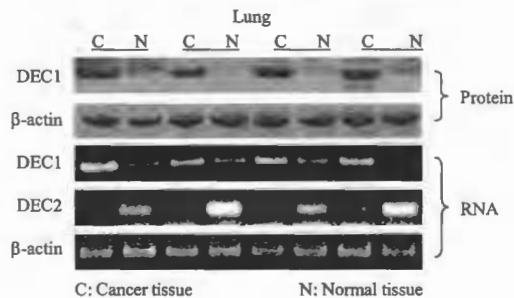
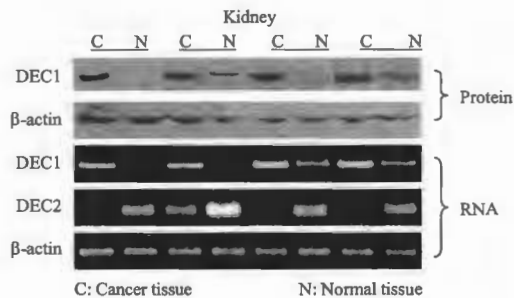
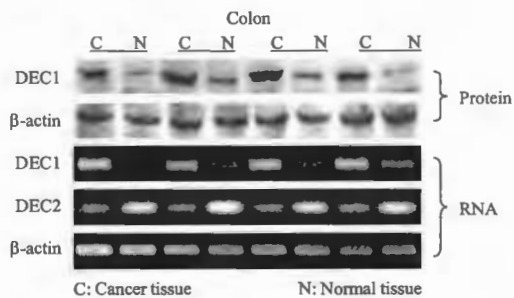
We have reported that DEC1 is abundantly expressed in colon carcinoma but not in the adjacent normal tissues (Li et al, 2002). The initial focus of the present study was to extend the expression study on DEC1 and to determine whether DEC1 and DEC2 shared similar expression patterns among paired cancer-normal tissues from the colon, kidney and lung. RT-PCR experiments with primers specific to DEC1 and DEC2 were performed. As shown in Figure 3-1, 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

Figure 3-1. Inversed expression patterns of DEC1 and DEC2 in the carcinoma and the adjacent normal tissues from the colon, kidney and lung

Total RNA (5 µg) of carcinoma-normal paired samples from the colon, kidney and lung was subjected to RT-PCR analyses with a ThermoScript I kit. For PCR amplification, a master tube containing all common reagents was prepared and equally distributed to individual PCR reaction tubes (DEC1, DEC2 and β-actin). PCR amplification was conducted with cycling parameters as follows: 95°C for 30s, 52°C for 30s and 68°C for 30 or 40s for a total of 32 cycles. The PCR-amplified products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. For Western blotting analysis, homogenates (10 µg) were subjected to SDS-PAGE. The immunoblot was incubated with the antibody against DEC1. The primary antibody was then located by horseradish peroxidase-conjugated goat anti-rabbit IgG and visualized with chemiluminescent substrate.



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 (Li et al, 2002). As expected, addition of tetracycline caused a concentration-dependent increase on the levels of DEC1 as determined by Western blots (Top, Figure 3-2A). Consistent with the inducible increase on 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 (Figure 3-2A). However, such inversed expression patterns were observed only in the cells expressing wild-type DEC1 (Figure 3-2A) but not the cells expressing the DEC1 mutant, even though the levels of DEC1-M were markedly induced by tetracycline (Figure 3-2B).

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 (Littlewood and Evan, 1998). A series of 5' deletion mutants of this reporter were also prepared and designed to specify the location of DNA sequence that is

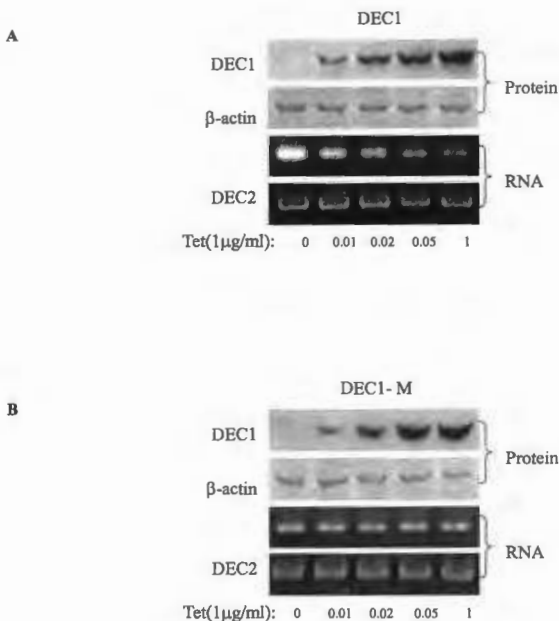


Figure 3-2. Repressed expression of DEC2 by DEC1 in stable transfected cells Stable transfected cells by DEC1 (A) or DEC1-M (B) were seeded in 6-well plate. After reaching ~80% confluence, cells were treated with tetracycline at various concentrations (0-1 μg/ml) for 24 h. Total RNA and homogenates were prepared and analyzed for the expression of DEC1 by Western blots or DEC2 by RT-PCR as described in the legend for Figure 3-1. Similarly the expression of β-actin was determined and served as controls.

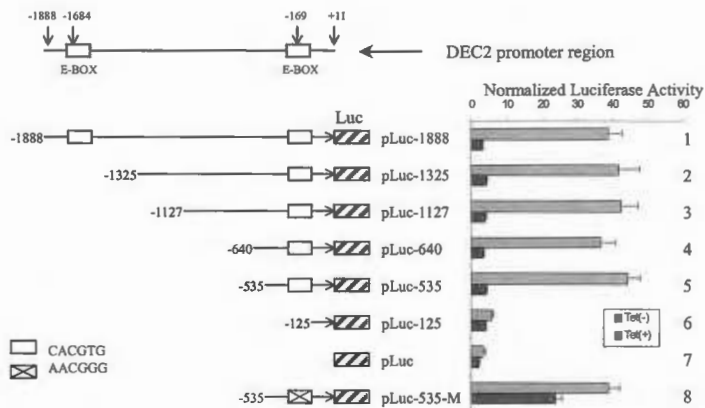
targeted by DEC1 (Figure 3-3A, Left). Co-transfection experiments were conducted to test these reporters for their ability to support DEC1-mediated activity. The stable transfected line (wild-type DEC1 only) was transfected again with a reporter construct and cultured in the presence or absence of tetracycline 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 Figure 3-3A (Right), 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 the 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 likely targeted by DEC1, a reporter with this E-box disrupted was tested for the ability to confer basal transcription. Reporter pLuc-535 was subjected to site-directed mutagenesis to selectively disrupt the E-box (CACGTG to AACGGG). Similarly, co-transfection experiments were performed. As shown in Figure 3-3A (Bottom), disruption of this E-box (pLuc-535-M) caused little changes on the basal activity (cultured without tetracycline), suggesting that this E-box contributes little to basal

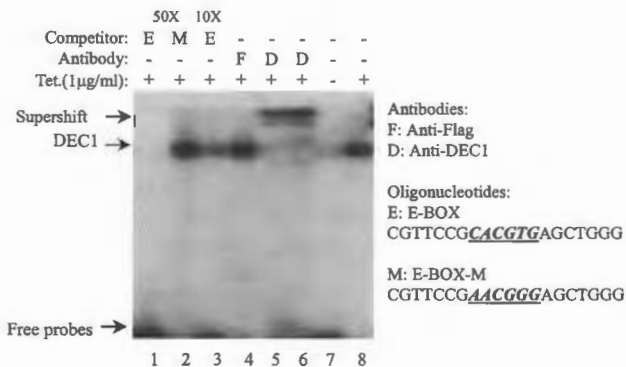
Figure 3-3. DEC1-mediated repression on the DEC2 promoter reporter and binding to the proximal E-box

(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 re-transfected cells were cultured in the presence or absence of tetracycline (1 µg/ml) for 24 h. The cells were collected, washed once with PBS 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 1X DNA binding buffer. For competition experiments, nuclear extracts were first incubated with excess cold probe (50 X in lane 1 or 10 X lane 3) and then mixed with the radiolabeled probe. Oligonucleotides (M) with the E-box disrupted were also used in the competition assays (50 X in lane 2). For super-shift 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.

A



B



transcription. In contrast, the reporter mutant (pLuc-535-M) exhibited only ~35% repression in response to DEC1 (Figure 3-3A, lane 8), which contrasts strikingly to 90% repression observed with the corresponding non-mutagenic reporter (Figure 3-3A, lane 5). These findings suggest that the proximal E-box is largely responsible for DEC1-mediated repression. It should be emphasized that similar observation was made with a substitution mutant reporter prepared from the longest reporter pLuc-1888, and the expression levels of DEC1 were comparable among all cells as determined by Western blots (data not shown).

We next examined whether this E-box interacted directly with DEC1. The DEC1 stable line was cultured in the presence or absence of tetracycline and nuclear extracts were prepared. 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 Figure 3-3B, 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 X (lane 1) or partially by 10 X excess cold probe (lane 3). However, the oligonucleotides (50 X) that harbored a mutated E-box (E-box-M) showed no competitive activity (lane 2). In addition, the shifted band was super-shifted by the anti-DEC1 but not the anti-Flag antibody. The super-shifted band appeared no matter if 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 C-terminal sequence of DEC1).

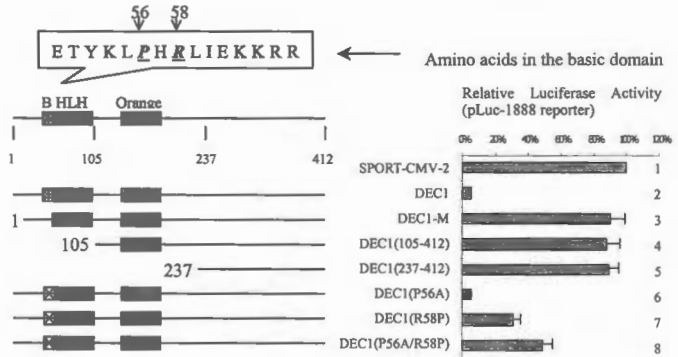
DNA binding is required to effectively repress the DEC2 promoter reporter

Disruption of the proximal E-box caused drastic but incomplete loss of responsiveness to DEC1 (Figure 3-3A), suggesting that DNA binding is not the only mechanism involved in DEC1-mediated repression on the DEC2 reporter or 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 (Figure 3-4A). A total of three deletion mutants (DEC1-M, DEC1₁₀₅₋₄₁₂ and DEC1₂₃₇₋₄₁₂) were prepared and all of them lacked the DNA binding domain. As shown in Figure 3-4A, additional sequences were also deleted in DEC1₁₀₅₋₄₁₂ (the HLH motif) and DEC1₂₃₇₋₄₁₂ (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 (Littlewood and Evan, 1998). Similarly three substitution mutants were prepared including DEC1_{P56A}, DEC1_{R58P} and DEC1_{P56A 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 (Littlewood and Evan, 1998). However, there is a major difference regarding the location of this proline. In other bHLH proteins, the proline is located 2 residues more carboxylic terminal (corresponding to residue 58 in DEC1)

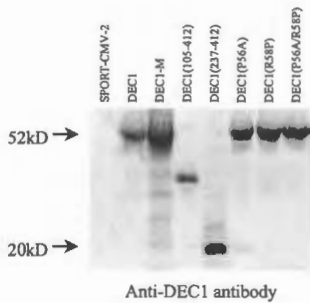
Figure 3-4. Essentiality of DNA binding for DEC1 to repress DEC2 promoter activity

(A) Co-transfection experiment Cells (293T) were cultured in 24-well plates and transiently transfected with DEC1 or a DNA binding defective mutant (100 ng), DEC2 promoter reporter (pLuc-1888, 100 ng) and the pRL-TK *Renilla* (1 ng). After a 24 h-incubation, cells were collected and analyzed for luciferase activities. Similarly firefly luminescence signal was normalized based on the *Renilla* luminescence signal, and the ratios from the cells transfected with the vector were calculated as 100%. *(B) Immunoblotting analysis* The cell lysates (10 μ g) from the cells used for reporter activity were analyzed for the expression of DEC1 or its mutants by anti-DEC1 antibody as described in the legend for Figure 3-1. *(C) EMSA* Nuclear extracts were prepared from cells transiently transfected with DEC1 or a mutant and incubated with the radiolabeled proximal E-box probe. The DNA-protein complexes were resolved by PAGE.

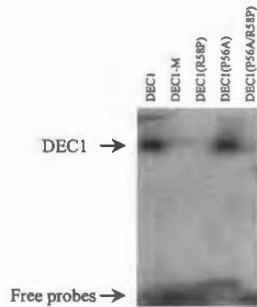
A



B



C



(Boudjelal et al, 1997; Fujimoto et al, 2001). Therefore, the mutants represented substitution of proline 56 with an alanine (DEC1_{P56A}), arginine 58 with a proline (DEC1_{R58P}), or both (DEC1_{P56A/R58P}).

Co-transfection experiments were conducted to test these DEC1 mutants for their ability to repress the DEC2 reporter (pLuc-1888). As shown in Figure 3-4A (Top), all deletion mutants (DEC1-M, DEC1₁₀₅₋₄₁₂, and DEC1₂₃₇₋₄₁₂) 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 DEC1_{P56A} mutant showed a similar potency as the wild-type DEC1 (~90% repression), whereas the other two mutants (DEC1_{R58P} and DEC1_{P56A/R58P}) caused only ~65 and ~50% repression, respectively. The expression of DEC1 and its mutants was comparable with an exception of DEC1-M that was expressed to a higher extent (Figure 3-4B), excluding the possibility that lack of expression was a contributing factor to the observed less repression by some of the mutants (e.g., DEC1_{P56A/R58P}). In order to determine whether these mutants, particularly the mutants DEC1_{R58P} and DEC1_{P56A/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, DEC1₁₀₅₋₄₁₂, and DEC1₂₃₇₋₄₁₂) showed no DNA binding activity (result shown on DEC1-M only) (Figure 3-4C). In contrast, DNA binding activity varied among the substitution mutants. DEC1_{P56A}

showed a similar binding ability as DEC1, whereas DEC1_{R58P} and DEC1_{P56A R58P} had no DNA binding activity, consistent with the fact that DEC1_{P56A} was the only substitution mutant that effectively repressed the promoter activity of DEC2 (Figure 3-4A).

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 C-terminus (Figure 3-5A). These C-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 Figure 3-5A, deletion of the C-terminal 65 residues (Flag-DEC1₁₋₃₄₇) caused no changes on the repressive activity (Littlewood and Evan, et al, 1998). In contrast, deletions of additional C-terminal sequence caused a partial or a complete loss of repressive ability. As a matter of fact, Flag-DEC1₁₋₁₅₀ no longer had any repressive activity. Western analyses were performed to confirm that the mutants were actually expressed slightly higher than the wild type DEC1 (Figure 3-5B).

Next, we examined whether these mutants actually retained DNA binding activity. EMSA was

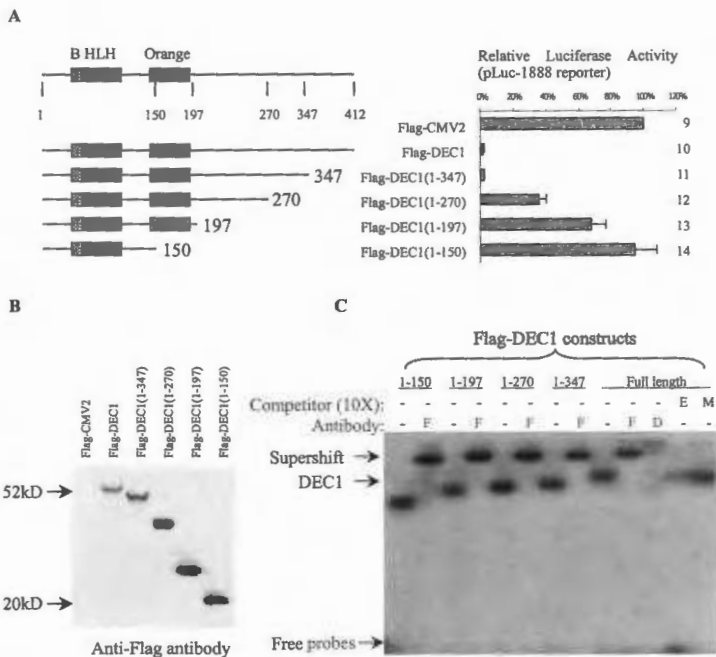


Figure 3-5. DNA binding is not sufficient for DEC1 to repress DEC2 promoter activity

(A) Co-transfection experiment Cells (293T) were cultured in 24-well plates and transiently transfected with DEC1 or a C-terminal truncated 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 for Figure 3-4. **(B) Immunoblotting analysis** The cell lysates (10 μ g) from the cells used for reporter activity were analyzed for the expression of DEC1 or its mutants by an anti-Flag antibody as described in the legend for Figure 3-1. **(C) EMSA** Nuclear extracts were prepared from cells transiently transfected with DEC1 or a mutant and incubated with radiolabeled oligonucleotides harboring the proximal E-box. Similarly competition experiments were performed with excess cold probe (E) or a mutant probe (M) as described in the legend in Figure 3-3B. For super-shift assays, an anti-Flag (F) or the anti-DEC1 (D) was added to the incubation mixtures before being analyzed by PAGE.

performed with the nuclear extracts from the cells used for reporter assays. As shown in Figure 3-5C, a shifted band was detected with all C-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, 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 (Figure 3-6A), although they all

shared two important features: lacking the entire DNA binding domain and lacking repressive activity themselves (Figure 3-4A). Among the substitution mutants, DEC1_{R58P} and DEC1_{P56A R58P} but not DEC1_{P58A}, partially but significantly reversed DEC1-mediated repression, consistent with the fact that DEC1_{P58A} was a potent repressor itself (as potent as wild type DEC1), whereas DEC1_{R58P} and DEC1_{P56A R58P} were much less repressive (Figure 3-4A). 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., DEC1_{P56A R58P}), otherwise, an additional band (e.g., DEC1₁₀₅₋₄₁₂) was detected if a mutant and DEC1 were electrophoretically distinct.

We also tested all C-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 (Figure 3-6B). 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 (Figure 3-4A) but completely reversed DEC1-mediated repression (Figure 3-6B). Among all mutants that were less repressive than wild type DEC1, only DEC1₁₀₅₋₄₁₂ and DEC1₂₃₇₋₄₁₂ failed to reverse the repression by DEC1 (Figure 3-6), and they were the only mutants that did not contain the HLH domain (Figure 3-4A and 3-5A), suggesting that the dominant interfering

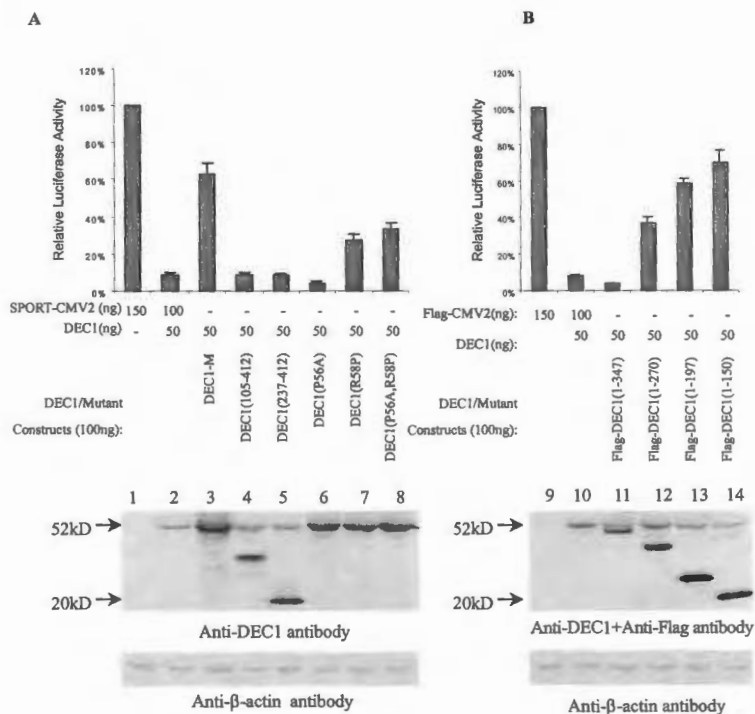


Figure 3-6. Dominant interfering regulation on DEC1-mediated repression

(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. Cell lysates (10 μ 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 C-terminus of DEC1). **(B) Effects of the C-terminal truncated mutants on the repressive activity by DEC1** The co-transfection and immunodetection were performed as described in the legend for Figure 3-6A. However, both anti-DEC1 and anti-Flag antibodies were simultaneously used for the immunodetection because the C-terminal truncated constructs were prepared with the Flag-CMV vector..

regulatory activity is achieved through the HLH domain. The HLH domain is known to mediate dimerization (1), mutants with an intact HLH domain likely 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 DEC1_{R58P} and DEC1_{P56A_R58P} (DNA binding defective mutants) markedly abolished the DNA binding ability of DEC1 when cells were co-transfected together with DEC1 and DEC1_{R58P} or DEC1_{P56A_R58P} (data not shown).

DISCUSSION

The bHLH proteins are intimately associated with developmental events such as cell differentiation and lineage commitment (Littlewood and Evan, 1998). 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 factors (Boudjelal et al, 1997; Fujimoto et al, 2001; Rossner et al, 1997; Shen et al, 1997). These proteins are shown to play important roles in cell differentiation, regulation of molecular clock, immune response and xenobiotic response (Boudjelal et al, 1997; Shen et al, 1997; Miyazaki et al, 2002; Shen et al, 2002; Sun et al, 2001; Honma et al, 2002; Zawel et al, 2002). Recently we have reported that DEC1 is abundantly expressed in colon carcinomas, antagonizes serum deprivation-induced apoptosis and selectively inhibits the activation of procaspases (Li et al, 2002). 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 (>90%) in the DNA binding region, whereas much diverse in other areas (<40%), our findings described in this report provide an important mechanism by which the cellular function of target genes likely shared by these proteins can be coordinately affected by members within the same class.

DNA binding is likely the primary mechanism responsible 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) (Sun and Taneja, 2000; Garriga-Canut et al, 2001; Yun et al, 2002). 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 repression by DEC1, this E-box exhibits a high affinity toward DEC1 and disruption of this E-box markedly reduces its responsiveness to DEC1 (Figure 3-3), suggesting that DNA binding is involved in the DEC1-mediated repression. Second, DEC1 deletion mutants (DEC1-M, DEC1₁₀₅₋₄₁₂, and DEC1₂₃₇₋₄₁₂), which lack the entire DNA binding domain, show neither DNA binding ability nor repressive activity (Figure 3-4A), providing direct evidence that DNA binding is required for DEC1 to repress the DEC2 promoter. Finally, among the substitution mutants, DEC1_{P58A} binds to the E-box as much as wild type DEC1 and is equally active in repression, whereas DEC1_{R58P} and DEC1_{P56A R58P} show no DNA binding ability and are much less repressive (Figure 3-4A), further supporting the notion that DEC1-mediated repression is largely achieved through DNA binding. The precise mechanism remains to be determined on whether DEC1_{R58P} and DEC1_{P56A R58P}, although lacking DNA binding ability, cause some repression. It is likely that these two mutants retain some DNA binding ability within the cells, but the conditions employed for EMSA fail to support such interactions. Alternatively, they exert repression through non-DNA binding mechanisms (Sun and Taneja, 2000; Garriga-Canut et al, 2001; Yun et al, 2002).

DNA binding, although required to exert effective repression, is not sufficient to repress the DEC2 promoter. Mutant Flag-DEC1₁₋₁₅₀, for example, binds effectively to DNA but shows no repressive activity (Figure 3-5A). 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 (Figure 3-5A). In this region, several helical structures and particularly an orange domain are located (Boudjelal et al, 1997; Fujimoto et al, 2001). These structures are assumed to mediate protein-protein interactions based on studies with other bHLH proteins (Littlewood and Evan, 1998; Boudjelal et al, 1997; Fujimoto et al, 2001). 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 DEC1₁₀₅₋₄₁₂ contain the entire sequence of this region but show no dominant interfering activity against wild type DEC1 (Figure 3-6A), suggesting that this region has intrinsic repressive activity. Alternatively, proteins assumed to be recruited are abundantly expressed in the cells employed in this study. Although we have not provided sufficient data to support protein recruitment in repressing DEC2, it can not be excluded that such events are involved in the regulation of other genes by DEC1, particularly given the fact mouse STRA13, a highly identical protein to DEC1, has been shown to interact directly with TFIIB through this region (Sun and Taneja, 2000).

DEC/STRA/SHARPs differ significantly from other bHLH proteins in terms of binding to DNA. Most bHLH proteins bind to E-box (CANNTG) or N-box (CANNAG). Binding

preference is specified by the sequence in the basic region. Generally, proline-containing basic regions have higher affinity toward the N-box (Littlewood and Evan, 1998; Akazawa et al, 1992), whereas the basic regions without a proline preferentially recognize the E-box. DEC/STRA/SHARPs contain a proline, however, this proline (residue 56 based on DEC1) is located 2 residues more amino terminal (Fujimoto et al, 2001). Instead, DEC/STRA/SHARPs have an arginine (residue 58) that substitutes the conserved proline among N-box binding bHLH proteins. Although initial studies suggest that STRA13 has no binding activity toward classic E- or N-box (Boudjelal et al, 1997), PCR-based site selection experiments have recently identified a class B type E-box (CACGTG) that is effectively bound by DEC1 and STRA13 (Zawel et al, 2002; St-Pierre et al, 2002). In this study, we have demonstrated that the contribution of Pro₅₆ to DNA binding is insignificant because mutant DEC1_{P58A} is equally effective as wild type DEC1 in DNA binding (Figure 3-4C). In contrast, introduction of a proline by substituting Arg₅₈ completely eliminates DNA binding activity (Figure 3-4C), suggesting that residue in this location is indeed important for E-box binding. It would be interesting to test whether DEC1_{R58P} 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, Figure 3-3A), 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 intracellular DNA binding. In support of this possibility, STRA13 has been shown to

preferably bind to an E-box flanked with certain nucleotides.

DEC1 and DEC2 share the DNA binding domain with an exception of a single residue (aspartate *versus* glutamate, the farthest N-terminal residue of this domain) (Fujimoto et al, 2001), therefore, DEC2 likely acts as an auto-regulator. This possibility is further supported by their highly identical sequences flanking the DNA binding domain. Immediately the C-terminal to the DNA binding domain is the helix-loop-helix domain that is identical between DEC1 and DEC2, and the N-terminal to this domain is an acidic residue-rich stretch in both proteins (Fujimoto et al, 2001). The DNA binding domain and its highly identical flanking sequences suggest that DEC1 and DEC2 have overlapping target genes, particularly those that are regulated through direct DNA binding. In support of this notion, mouse proteins (STRA13 and DEC2) have been recently shown to repress Clock/Bmal1-induced activation of the *Per* promoter (Honma et al, 2002), a gene that is involved in the regulation of molecular clock. Therefore, it is likely that DEC/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 (Sun et al, 2001). It should be emphasized, however, that DEC1 and DEC2 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 C-terminal half and exhibit several major structural differences (Fujimoto et al, 2001). Both

DEC1 and DEC2 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 DEC2 but absent in DEC1. 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 (Figure 3-5A.). Amino acid repeats, on the other hand, are implicated in protein folding, protein-protein interactions and degradation (Katti et al, 2000).

DEC1-mediated repression is likely responsible for the differences on cell and tissue distributions between DEC1 and DEC2. Although Northern analyses have shown that DEC1 and DEC2 have an overlapping tissue distribution (Fujimoto et al, 2001; Shen et al, 1997), it remains to be determined whether they are actually expressed in the same cell type and to a similar extent (Fujimoto et al, 2001; Shen et al, 1997). Some organs with high levels of DEC1 (e.g., liver) express lower levels of DEC2 (Shen et al, 1997). Very recently, DEC1 and DEC2 are found to regulate the mammalian molecular clock, but they exhibit distinct and area-dependent expression patterns in the brain (Sun et al, 2001). In this report, we have demonstrated that these two proteins exhibit inversed expression patterns among the paired tumor-normal tissues, forced expression of DEC1 causes proportional decreases in the expression of DEC2 (Figure 3-1 and 2), providing direct evidence that increased expression of DEC1 is at least in part responsible for decreased expression of DEC2 in a given cell context.

DEC1-mediated repression, although profound, may not always dictate the expression of DEC2. For example, DEC1 and DEC2 are both up-regulated in response to hypoxia induction (Miyazaki et al, 2002). Acute hypoxia is considered severe cytotoxic stimulus, rapid induction of both genes maximizes cellular survival mechanism based on our recent report that DEC1 is antiapoptotic (Li et al, 2002), although it remains to be determined whether DEC2 is actually antiapoptotic as well.

In summary, we have demonstrated that DEC1 is a negative regulator on the expression of DEC2. These two proteins exhibit inversed expression patterns among paired samples from the colon, kidney and lung. An inducible expression system demonstrates that increased expression of DEC1 proportionally decreases the expression of DEC2. The DEC1-mediated repression is primarily achieved by binding to the E-box in the proximal promoter of DEC2. Site-directed mutagenesis studies show that arginine-58 in the DNA binding domain is critical for DEC1 to interact with this E-box. Given the fact that DEC/STRA/SHARP proteins are being emerged as very important regulators in a vast array of cellular events including cell differentiation, maturation of lymphocytes, oncogenesis, molecular clock and xenobiotic response, our findings described in this study provide an important mechanism that these proteins regulate the cellular function not only by modulating the expression of their target genes but also the expression of the members within the same class.

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CONCLUSIONS

The purpose of this dissertation is to elucidate the oncogenic roles of DEC1 and determine the molecular actions of DEC1 on transcription regulation. Unique structure of DEC1 indicates that it has distinct biological functions and unique mechanism on transcription regulation. Tumor-related expression pattern of DEC1 suggests that DEC1 is involved in oncogenesis. Thus, the significance of the studies reported in this dissertation will contribute to our basic understandings of bHLH proteins as a family of transcription factors involved in oncogenesis.

Expression of DEC1 is related to tumor and cell cycle progression.

In an effort to identify genes that are differentially expressed between colon carcinomas and the adjacent normal tissues, we performed cDNA subtractive assay, a technique that identifies genes highly expressed in one tissue over another. A cDNA with 98% sequence identity to DEC1 was identified. The amino acid sequence encoded by this cDNA was exactly the same as DEC1. The initial goal of the studies described in manuscript I was to determine if the expression of DEC1 is related to tumors. I hypothesized that DEC1 is up-regulated in tumor tissues. The rationale for this hypothesis was based on the following observations: 1) DEC1 is cloned from human colon carcinoma tissues, which indicates that DEC1 might be highly expressed in tumor tissues; 2) cells that lack the functional tumor suppressor VHL express higher levels of DEC1; 3) hypoxia induces DEC1 via HIF, a transcription activator which is highly expressed in tumor.

To examine the relationship between the expression of DEC1 and tumor, human paired samples from carcinoma tissues and adjacent normal tissues were collected from colon, lung and kidney. Both mRNA levels and protein levels of DEC1 were markedly higher in all these carcinoma samples, but very low levels of DEC1 were detected in the paired normal samples. This result confirmed our hypothesis that DEC1 is highly expressed in tumor tissue.

Since DEC1 is abundantly expressed in tumors but not in the adjacent normal tissues, its expression is probably related to nutrient requirement and cell confluence. In DLD cells, a colon carcinoma derived human cell line, post-confluent cells express much higher DEC1 than less confluent cells. post-confluent cells usually undergo growth arrest and fail to continue cycle progression. Therefore, DEC1 expression is probably linked to cell cycle states. To test this possibility, DLD cells were treated with different cell cycle blockers to arrest cells at G0, G1, S and G2 phases. Protein level of DEC1 demonstrated that S blocker hydroxyurea and G2 blocker nocodazole markedly induced DEC1. The induction was observed as early as 6 hours after treatment. Cell cycle synchronization normally needs 24 hours. This rapid induction of DEC1 suggests that the induction of DEC1 occurs prior to cell arrest rather than as a result of this effect. Therefore, DEC1 may slow down cell cycle progression and has proliferation-inhibitory effects.

In order to test the possibility that DEC1 inhibits cell proliferation, stable transfectants were

prepared. Sense, mutant and anti-sense DEC1 constructs were stably transfected into T-Rex 293 cells, in which the inserted genes can be inducibly regulated by tetracycline. As expected, proliferation studies revealed that sense cells with tetracycline showed much lower growth rates than the same cells cultured without tetracycline. The mutant cells, which encode a mutant DEC1 lacking the DNA binding domain, had no effects on proliferation. The requirement for DNA binding domain implied that DEC1 inhibits cell proliferation through regulating other protein expressions. It is interesting to note that DEC1 has proliferation inhibiting activity. Cancer cells always have high proliferation rate. However, in some conditions such as in short of blood supply, high proliferation is not good for cell survival. Cells with lower growth rate may have higher survival ability and are resistant to death. Thus, DEC1 might help cells against apoptosis, an event occurs when cells are in short of nutrients.

DEC1 antagonizes serum deprivation-induced apoptosis.

Tumor related expression of DEC1 and its anti-proliferation activity suggests it may protect cells against apoptosis. Therefore, another goal of the studies described in manuscript I was to test the hypothesis that DEC1 has antiapoptotic activity, an effect that favors tumor survival. The rationale for this hypothesis included: 1) DEC1 is highly expressed in over-confluent cells which usually undergo apoptosis due to nutrient depletion; 2) DEC1 is rapidly induced by detrimental stimuli like hypoxia and treatment with cytotoxic chemicals like hydroxyurea and nocodazole, indicating that DEC1 has cytoprotective functions and prevents cells from death; 3)

DEC1 is up-regulated by HIF-1 α , a transcription factor that has been shown to have anti-apoptotic effects.

To test this hypothesis, the DEC1 stable transfectants were cultured in serum free media in the presence or absence of tetracycline to imitate nutrient depletion conditions and to induce apoptosis. After 48 hours, cells cultured without tetracycline showed typical apoptotic changes while cells cultured with tetracycline displayed normal appearances. This anti-apoptotic effect was also confirmed by DNA fragmentation, a hallmark of cell apoptosis. Like anti-proliferation effects, this antiapoptotic effect also required the DNA binding domain because DEC1 mutant cell lines failed to prevent serum deprivation induced apoptosis.

Caspases are a family of cysteine proteases that play central roles in regulating the apoptotic process. Pro-apoptotic signals, like FasL, TNF and DNA damage activate initiator caspase 8. Cytochrome C released from mitochondria is coupled to the activation of caspase 9, another key initiator caspase. Activation of both caspase 8 and 9 results in the activation of downstream effector caspases 3 and 7, which in turn cleaves cytoskeletal and nuclear proteins and induces apoptosis. To test whether DEC1 has any effects on these caspases activation, cell lysates from serum deprived stable transfectants were prepared and assayed for the activities of caspase 3/7, 8 and 9. The activities of caspase 3/7 and 9, but not 8, were markedly decreased by DEC1. The expression levels of these procaspases have no changes, which implied that DEC1 inhibits the

activation process of procaspases rather than represses the expression level of procaspases.

DEC1 upregulates the antiapoptotic protein, survivin.

The goal of the studies reported in manuscript II was to extend the apoptotic study of DEC1 and determine the signaling pathways leading to apoptotic inhibition. Several classes of proteins, notably the inhibitor of apoptosis protein (IAP) family, have been shown to inhibit procaspase activation and the catalytic activity of several caspases. To test whether DEC1 has any effects on IAP protein regulations, we examined the expression levels of several IAP proteins including cIAP-1, cIAP-2, XIAP and survivin in DEC1 stable transfectants treated with serum deprivation. Among these IAP proteins, only survivin was markedly increased in tetracycline-treated cells, suggesting that DEC1 selectively increases the expression of IAP proteins. The induction of survivin by DEC1 was consistent to the following observations: 1) Both survivin and DEC1 are upregulated in tumors; 2) The expressions of survivin and DEC1 are cell cycle dependent and upregulated by cell cycle blocker nocodazole; 3) Survivin has been shown to inhibit the activation of procaspase 3 and 9, but not 8, consistent to the activities of DEC1. Moreover, paralleled expression pattern between DEC1 and survivin in paired tumor-normal tissues also supported the notion that DEC1 regulates the expression of survivin.

It should be noted that the upregulation of survivin by DEC1 only occurred in serum deprived cells and was time-dependent. Time course studies showed that survivin expression declined

after 24 hours of serum deprivation, but little changes were observed before 24 hours incubation. However, the expression of DEC1 prevented the decrease of survivin expression and kept survivin to a relative high level, thus protected cells from death.

Two mechanisms, namely increased survivin stability and/or expression, are likely responsible for such a relatively higher steady level of survivin. Same increase patterns were observed in both survivin mRNA and protein levels, suggesting that DEC1 mediated induction of survivin is achieved through transcription activation. To test this possibility, transfection studies were conducted with survivin promoter reporters pSurvivin-6270 and pSurvivin-268. Both these two reporters showed 5 fold induction of survivin by DEC1 in serum deprived stable transfectants. Similar induction fold on reporter pSurvivin-6270 and pSurvivin-268 indicated that the responsible sequences for DEC1 are located downstream of site -268 in survivin promoter.

Multiple Sp1 binding sites are responsible for the survivin induction by DEC1

Sequences analysis reveals that a clustered Sp1/CDE region (cycle dependent element) is located in the proximal region of survivin promoter and has been shown to mediate basal transcription. However, no E-box is identified in this region. In order to identify DNA sequences that act as potential binding sites for DEC1, ten oligonucleotides were synthesized to span the entire promoter region of reporter pSurvivin-268. EMSA studies with these

oligonucleotides identified Sp1-127 and Sp1-226 as two Sp1 binding sites whose DNA-protein bindings were dramatically increased in the presence of DEC1. Deletion and substitution mutation assays on pSurvivin-268 reporters showed that Sp1 sites-127 and 226 additively activated the survivin promoter reporter in response to DEC1. Super-shift studies using anti-Sp1 and anti-DEC1 antibodies found that both DEC1 and Sp1 bond to the Sp1 sites-127 and 226. The DEC1 mutant lacking the DNA-binding domain failed to induce survivin, suggesting the DNA binding requirement of this activity. The delayed induction on survivin implied that the induction was through regulating other transcription factors. However, the direct binding of DEC1 to Sp1-protein complex indicated that DEC1 was directly involved in this transcription activation. Therefore, DEC1 up-regulates survivin probably through more than one mechanism. It remains to be determined whether the interaction between Sp1-binding site and DEC1 requires basic domain and whether DEC1 binds to Sp1-binding site directly or through other proteins.

DEC1 negatively regulates the expression of DEC2

Soon after DEC1/STRA13/SHARP2 was reported, Fujimoto et al (2001) identifies human, mouse and rat DEC2, a novel member of the DEC subfamily. DEC2 has high (97%) similarity with DEC1 in the bHLH region but has a quite different c-terminal part. These two proteins show different tissue distributions. To determine if DEC2 has similar expression patterns in tumors like DEC1, mRNA levels of DEC2 were tested in paired tumor-normal tissues from the

colon, lung and kidney. In contrast to DEC1 which was highly expressed in tumors, DEC2 was expressed markedly higher in the adjacent normal tissues and had reverse expression patterns with DEC1 in all the samples tested. Searching of the DEC2 promoter sequence identified two classical E-box sequence CACGTG at position -1684 and -169. Zawei et al (2000) reports that DEC1 bind to E-box and mediates transcription repression. But Boudjelal et al (1997) shows that DEC1 failes to bind either E-box or N-box. To solve this controversy, the goal of the studies described in manuscript II was to determine whether DEC1 binds directly to E-box sequence in the promoter region of DEC2 and represses its transcription.

In DEC1 stable cell lines, forced expression of DEC1 proportionally decreased the expression of DEC2. This result strongly supported our hypothesis that DEC1 repressed the expression of DEC2. However, the repression on DEC2 was only observed in DEC1 sense lines, but not in DEC1 mutant lines, cells that express a mutant DEC1 lacking the DNA binding domain. The requirement of DNA binding domain suggests that the repression on DEC2 is through a DNA binding mechanism. To test this possibility, a DEC2 promoter reporter (pLuc-1888) was constructed to contain the promoter region (-1888 to +11) of DEC2 gene. Co-transfection and luciferase assays showed that DEC1 repressed the transcription of pLuc-1888 reporter by as much as 90%. A 5' deletion mutant reporter pLuc-535 gave the same result as pLuc-1888, which indicated that the repression was achieved through E-box -169 rather than E-box -1684. Disruption of E-box -169 by site-directed mutagenesis in pLuc-535 abrogated the repression

by 60%. EMSA studies gave direct evidence that DEC1 bond to the E-box -169 and the specificity of this binding was confirmed by super-shift experiment. Taken together, these results suggests that DEC1 represses the transcription of DEC2 and the repression is primary achieved through direct DNA binding to the E-box in the proximal promoter of DEC2.

To further expand our studies on molecular mechanisms of DEC1-mediated transcription repression, many DEC1 mutants were included in the reporter assay. Substitution mutation of arginine 58 to proline totally abrogated the E-box binding ability of DEC1 and reduced the transcription repression by 60%, which pointed out the importance of this residue in DNA binding. C-terminal deletion mutants of DEC1 showed various transcription repression and indicated that deleted region from residue 150 to 347 were important for the repressive activity of DEC1. The 150-347 region either has intrinsic repressive activity, or is responsible for recruiting transcription co-repressors. The exact mechanisms still need to be clarified.

In conclusion, the studies reported in this dissertation demonstrate that DEC1 is highly expressed in tumor and inhibits cell proliferation. Expression of DEC1 protects cells against serum deprivation induced apoptosis through upregulation of anti-apoptotic protein survivin. The upregulation of survivin is regulated by two Sp1 binding sites in the proximal promoter of survivin. Both DEC1 and Sp1 are involved in the binding of these Sp1 sites. In contrast to transcription activation, DEC1 negatively regulates the expression of DEC2 through binding to

the E-box in the proximal promoter. These findings establish the oncogenic roles of DEC1 and elucidate the signaling pathway that leads to antiapoptotic activity. The studies on transcription regulations provide molecular mechanisms that DEC1 regulates the transcription as an activator as well as a repressor depending on the genomic context of a target gene.

APPENDICES

ABBREVIATION

ARNT: Ah receptor nuclear translocation protein

bHLH: basic helix-loop-helix

CHAPS: 3-[C(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid

DEC: differentially expressed in chondrocytes

DMEM: Dulbecco's modified Eagle's medium

DTT: dithiothreitol

E-box: Ephrussi box

EMC: extramacrochaetae protein

EMSA: Electrophoretic mobility shift assay

E(spl): enhancer of split protein

HAT: histone acetyltransferase

HES: hairy and enhancer of split-related mammalian proteins

HLH: helix-loop-helix

IAP: inhibitor of apoptosis protein

MAD: Max-associated dimer protein

Mash: mammalian Achaete-Scute homologue protein

MAX: Myc-associated x protein

MyoD: myogenic determination factor

PCR: polymerase chain reaction

PBS: phosphate-buffered saline

PMSF: phenylmethanesulfonyl fluoride

RT-PCR: reverse transcription-polymerase chain reaction

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SHARP: split and hairy related protein

STRA: stimulated with retinoic acid

TAL: T cell acute leukemia protein

TBP: TATA-binding protein

TFII: transcription factor II

TGF- β : transforming growth factor β

VHL: Von Hippel-Lindau

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