Abundant expression of Dec1/stra13/sharp2 in colon carcinoma: its antagonizing role in serum deprivation-induced apoptosis and selective inhibition of procaspase activation

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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, differentially expressed in chondrocytes/stimulated with retinoic acid/split and hairy-related proteins (DEC/STRA/SHARPs) represent a new class of bHLH proteins. The present study describes the functional characterization of DEC1. 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, which suggests that DNA binding is required for these actions. Enzymic assays and immunoblotting analyses demonstrated that induction of DEC1 by tetracycline significantly decreased the activation of procaspases 3, 7 and 9 but not procaspase 8. The selective suppression on the activation of procaspases 3, 7 and 9 over procaspase 8 suggests that DEC1-mediated anti-apoptosis is achieved by blocking apoptotic pathways initiated via the mitochondria. The results functionally distinguish DEC1 from other bHLH proteins and directly link this factor to oncogenesis.

Key words: basic helix–loop–helix, proliferation inhibition, transcription factor.

INTRODUCTION

The basic helix–loop–helix (bHLH) proteins belong to a family of transcription factors [1]. These proteins are known to play important roles in myogenesis (MyoD/E47) [2], neurogenesis (NeuroD) [3], cell proliferation and differentiation (Myc/Max) [4], sex determination (E12/Da/ESC) [5], regulation of immunoglobulin genes (TFEC/TFE3) [6], phospholipid metabolism (Ino2/Delila) [7] and xenobiotic response (AHR/ARNT) [8]. The bHLH proteins are structurally featured by their bHLH motif [1]. The bHLH motif is divided into two functional domains: the HLH domain and an adjacent basic region. The HLH domain, consisting of two-amphipathic α -helices connected by a non-conserved 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 [1,9,10].

Human protein differentially expressed in chondrocytes (DEC), mouse protein stimulated with retinoic acid (STRA) and rat split and hairy-related protein (SHARP) represent a new and structurally distinct class of bHLH proteins [11–14]. In each species, two related members are identified with a sequence identity of > 90% in the bHLH region and approx. 50% in total. Based on sequence alignment, DEC/STRA/SHARPs

closely resemble *Drosophila* Hairy and enhancer of split *E(spl)* as well as the mammalian homologues (e.g. Hes) [15–17]. They share the highest sequence identity (approx. 40%) in the bHLH domain. The basic region contains a proline residue usually located in repressive bHLH proteins [1]. Like Hairy/*E(spl)*/Hes, DEC/STRA/SHARPs contain an orange domain, which is approx. 30 amino acids away from the second helix of the bHLH motif and is supposed to mediate local repression of bHLH activators. In contrast with Hairy/*E(spl)*/Hes proteins, DEC/STRA/SHARPs lack the C-terminal WRPW motif. Through this sequence, Hairy/*E(spl)*/Hes recruit a co-repressor known as Groucho to the transcription regulatory complex [15,18]. Recruitment of Groucho is responsible for a vast array of biological activities of Hairy/*E(spl)*/Hes proteins including cellular differentiation and lineage commitment [15,18–20].

Lack of the functionally important WRPW motif suggests that DEC/STRA/SHARPs have rather distinct biological functions and act through different mechanisms. In a cultured cell system, mouse STRA13 has been shown to promote neuronal but repress mesodermal and endodermal differentiation [11]. NIH 3T3 cells transfected with STRA13 exhibit a reduction in colony-forming numbers compared with cells transfected with an empty vector [21]. Disruption of STRA13 gene results in defective T-cell activation and the genesis of autoimmune disorders in aging mice [22]. Two SHARP proteins of rat have distinct tissue distributions

Abbreviations used: bHLH, basic helix–loop–helix; DEC, differentially expressed in chondrocytes; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; *E(spl)*, enhancer of split; HIF-1, hypoxia-inducible factor 1; poly(A)⁺, polyadenylated; SHARP, split and hairy-related protein; STRA, stimulated with retinoic acid.

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but both are present in a subset of mature neurons and are drastically induced by neurotrophins and glutamatergic neurotransmission [13], 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 [21,23]. Human DEC1 is present in a wide range of tissues including some tumour tissues and tumour-derived cell lines [12,14,24,25]. Absence of the von Hippel–Lindau tumour suppressor proteins up-regulates the expression of DEC1 gene [24]. These findings suggest that DEC1 has both physiological and oncogenic significance.

The present study describes the functional characterization of DEC1. Subtractive experiments with matched samples, followed by Northern- and Western-blot analyses, 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 chemiluminescent substrate were from Pierce (Rockford, IL, U.S.A.). Substrates for caspases were from Biomol (Plymouth Meeting, PA, U.S.A.). Antibodies recognizing cleaved procaspases were from Cell Signaling. Dulbecco's modified Eagle's medium (DMEM) and PCR reagents were from Gibco. Unless otherwise indicated, all other reagents were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Plasmid

A cDNA encoding the full-length DEC1 was isolated by a cDNA-trapping method [26]. To establish stable transfectants, several DEC1 constructs were prepared with the tetracycline-inducible pcDNA4/TO vector. These constructs included sense, antisense and mutant DEC1. 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 (ccggaattcATCCAGA-CGCTCCGCTAGTG) and A-1 (ctgctctagaGCAGGAAGTAGCGAGGAAGG), 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 (CTCGTT-AATCCGGTCACGCTCCTTGCTCTCGCTCCG), whereas the C-terminal fragment was generated with primers S-2 (CG-GAGCGAGAGCAAGGAGCGTGACCGGATTAACGAG) 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 resulting 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 resulting 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. Samples were collected from two male and two female patients and their age was between 41 and 62. The size of tumours was approx. 5 cm in diameter and located at the sigmoid or left colon. The degree of differentiation of tumours 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 [27]. 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 dithiothreitol (DTT)]. The homogenates were centrifuged at 12000 *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 Laboratories, Palo Alto, CA, U.S.A.). Polyadenylated [poly(A)⁺] RNA was isolated by two cycles of oligo(dT)–cellulose column chromatography. The poly(A)⁺ RNA from four individual cancer tissues was pooled, so was the poly(A)⁺ RNA from the adjacent normal tissues. First-strand cDNA was synthesized with avian myeloblastosis virus 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 (tumour 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 tumour 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 tumour tissue than the adjacent normal tissues because only the cDNAs from the tumour 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, Madison, WI, U.S.A.). The resultant clones were isolated and subjected to sequencing analysis.

Subcellular localization of DEC1

Cells (HEK-293T) were plated on to 35 mm culture dishes in DMEM supplemented with 10% foetal calf serum at a density of 3×10^5 cells/dish. On day 2, when the cells reached approx. 70% confluence, they were transfected with DEC1 or the corresponding empty plasmid for 4 h with lipofectAMINETM (Gibco).

Thereafter, the media were replaced with fresh media. Transfected cells were cultured for an additional 48 h, washed once with PBS and scraped from the dish in PBS. They were pelleted by centrifugation at 3000 *g* and rapidly resuspended in three pellet volumes of the homogenization buffer [10 mM Hepes (pH 7.9)/1.5 mM MgCl₂/10 mM KCl]. The resuspended cells were then subjected to a glass Dounce homogenizer for 10 strokes. The cytoplasm and nucleus were separated by centrifugation at 3000 *g* for 15 min, and the nuclear pellets were resuspended in lysis buffer [homogenization buffer plus 0.5 % Triton X-100 and 0.5 % SDS]. Cell fractions were analysed for DEC1 abundance.

Stable transfection

Stable transfection was conducted with T-Rex-293 cell line, which was derived from the HEK-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 sense, antisense or mutant DEC1 (1 μ g) for 4 h by lipofectAMINETM. Transfected cells were cultured in full media for 24 h, and then split into fresh media. The split cells were seeded to 10 % confluence and cultured in media containing both ZeocinTM (300 μ g/ml) and blasticidin (5 μ g/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-blot analysis.

Proliferation assay

Stable transfectants expressing sense, antisense or mutant DEC1 were seeded in a 96-well plate (12 500 cells/well) and cultured in DMEM supplemented with foetal bovine serum (0–10 %) in the presence or absence of tetracycline (0–1 μ g/ml). After incubation at 37 °C for 48 h 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 (approx. 80 % confluence) were subjected to apoptotic induction by culturing in serum-free media in the presence or absence of tetracycline (1 μ g/ml). After a 48 h 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 % (v/v) Triton X-100/0.1 % SDS]. The high-molecular-mass DNA was removed by centrifugation at 14 000 *g* for 30 min. The supernatants were then sequentially extracted with equal volumes of a mixture of chloroform and phenol/chloroform/isoamyl alcohol (25:24:1). The soluble DNA was precipitated with 2.2 vols. of ethanol in the presence of 0.5 M NaCl. The DNA was resuspended with TE buffer and treated with DNase-free RNase (0.1 μ g/ μ l) for 3 h at 30 °C to remove RNA. DNA fragmentation was analysed by agarose-gel electrophoresis (1.5 %). Cell lysates were also prepared and analysed for the expression of DEC1. To quantitatively specify the degree of DNA fragmentation as a function of the expression levels of DEC1, identical experiments (the sense line only) were conducted but various concentrations (0–1 μ g/ml) of tetracycline were added to increase proportionally 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 caspases 3/7, 8 and 9 respectively. Stable transfectants were seeded at approx. 60 % confluence in 6-well plates. When the cells reached approx. 80 % confluence, the media were replaced with serum-free media and cultured in the presence or absence of tetracycline (1 μ g/ml) for 48 h. The cells were then harvested, washed with PBS and lysed on ice for 30 min in lysis buffer (100 μ l/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, 10 μ g/ml pepstatin and 10 μ g/ml leupeptin. The lysates were then subjected to centrifugation at 10 000 *g* for 1 min at 4 °C. To determine caspase activity, 100 μ l of supernatant (1 mg/ml) was mixed with 100 μ l of reaction buffer [20 mM Tris/HCl (pH 7.4), 4 mM EDTA, 0.2 % CHAPS, 0.1 mM PMSF, 1 mM DTT, 10 μ g/ml pepstatin and 10 μ g/ml leupeptin]. Colorimetric substrate was added to the mix at a final concentration of 200 μ M. After a 2 h 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.

Other analyses

Northern- and Western-blot analyses were conducted as described previously [28,29]. Preparation of an antibody raised against a peptide (CSQALKPIPLNLETKD) derived from DEC1 has been described previously [28]. Protein concentration was determined with bicinchoninic acid assay (Pierce). Data are presented as means $\pm$ S.D. 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 the two values are made with Student's *t* test at *P* < 0.05.

RESULTS

Molecular cloning and tumour-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 matching 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 [26]. The cDNA was 3361 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 [14]. 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-blot analyses. As shown in Figure 1(A), without exception, DEC1 gene was expressed abundantly in the colon adenocarcinomas (more than 20 times based on densitometrical analysis), but only little or very low levels of DEC1 mRNA were detected in the adjacent normal tissues. 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(B), this antibody detected a protein with a

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

(A) Total RNA (10 µg) from colon carcinoma and the adjacent normal tissues was subjected to agarose-gel electrophoresis and transferred on to a Nytran nylon membrane. The blots were detected with ³²P-labelled probes from cDNA encoding DEC1. To normalize the abundance of 28 S rRNA, the same membrane was reprobbed with a [³²P]ATP-labelled oligonucleotide hybridizing 28 S 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 raised against DEC1. The primary antibody was then located by alkaline phosphatase-conjugated goat anti-rabbit IgG. (B) Specificity of the prepared antibody. The plasmids harbouring the DEC1 cDNA (transfected) or no insert (control) were used to transfect HEK-293T cells and the subcellular fractions were prepared as described in the Materials and methods section. The fractions (5 µg) were subjected to SDS/PAGE and analysed for DEC1 expression.

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

Effects of cell confluence and cycle blockers on DEC1 expression

Given the fact that DEC1 is abundantly expressed in tumour but not in the adjacent normal tissues, its expression is probably linked to the cell proliferation state and cycle progression. 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 (1×10^5 to 8×10^5). On day 3, when wells with the top three seeding densities were confluent or post-confluent, the cells were harvested. By this time, the cells at the two lowest seeding densities were still rapidly proliferating. Total RNA and cell lysates were prepared and analysed for the expression of DEC1. As shown in Figure 2(A), 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 µg) 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 2 Regulation of DEC1 expression by cell cycle blockers

The abundance of DEC1 mRNA and proteins was determined by Northern- and Western-blot 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 (1×10^5 to 8×10^5). On day 3, total RNA and lysates were prepared and analysed for the expression of DEC1. (B) Effects of cell cycle blockers on DEC1 expression. Cells were seeded at a 30% confluence. After a 12 h incubation, media were replaced with cycle block media (G₀ block, 0.1% serum; G₁ block, amino acid-deficient media; S block, 3 mM hydroxyurea; G₂ block, 500 ng/ml nocodazole). After a 30 h-incubation, lysates were prepared and analysed for DEC1 expression. (C) Time course of hydroxyurea and nocodazole on DEC1 expression. Cells were treated as described in (B) and harvested at indicated times. Lysates were analysed by Western-blot analysis. Data are presented as representative results obtained in at least three independent experiments.

Confluent and post-confluent cells tend to have better cell-cell contact and usually undergo growth arrest with a lower number of cells continuing cycle progression. Therefore, confluence-induced expression of DEC1 is probably due to cell contact, growth arrest or both. To test these possibilities, cells were cultured at subconfluence in the presence of cell cycle blockers. For the G₀ block, cells were cultured in normal growth media but supplemented with only 0.1% serum. For the G₁ block, cells were cultured in amino acid-deficient media (no methionine and isoleucine). For the S block, cells were cultured in normal growth media containing hydroxyurea. For the G₂ block, cells were cultured in normal growth medium containing nocodazole. Cells were cultured in the cycle block media for 30 h, and lysates

Figure 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 $\mu\text{g/ml}$) for 24 h, and lysates were analysed 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 $\mu\text{g/ml}$) for 24 h. DEC1 induction was analysed as described in (B). The Figure shows representative results.

(10 μg of total protein) were prepared and analysed for the levels of DEC1. As shown in Figure 2(B), both hydroxyurea and nocodazole were shown to markedly induce DEC1. The time-course study showed that the induction was observed as early as 6 h after treatment (Figure 2C) and reached the maximum 30 h 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 (results 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, and therefore has proliferation-inhibitory effects. To test this possibility, sense, antisense and mutant DEC1 constructs (Figure 3A) were used to stably transfect T-Rex 293 cells, which allowed the inserted gene to be inducibly regulated by tetracycline. As shown in Figure 3(B), 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 3C).

To determine the effects of DEC1 on cell proliferation, the stable transfectants expressing sense, antisense or mutant DEC1

Figure 4 Effects of DEC1 on cell proliferation

Stable transfectants were plated in 96-well plates (12500 cells/well) and cultured in the presence or absence of tetracycline (Tet, 1 $\mu\text{g/ml}$) for 48 h. The proliferation rate was determined with a CellTiter kit by measuring the absorbance at 570 nm (Promega). To reflect precisely the proliferation rate, control plates were seeded similar to the experiment plates but the absorbance was determined 4 h 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 $\mu\text{g/ml}$). (B) Proliferation rate as a function of serum concentrations. (C) Proliferation rate as a function of tetracycline concentrations. Results were obtained for at least three independent experiments. *Significantly different from cells cultured without tetracycline.

Figure 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 approx. 40% confluence and grown in full media in the presence or absence of tetracycline (Tet, 1 µg/ml). After a 12 h incubation, the cells were cultured in serum-free media (tetracycline was kept the same). After a 48 h incubation, morphological changes were assessed ($\times 100$). (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 µg/ml tetracycline until reaching approx. 80% confluence. The media were then replaced by serum-free media (tetracycline was kept the same). After a 48 h incubation, DNA fragmentation was analysed by agarose-gel electrophoresis (1.5%). Cell lysates (5 µg) were also prepared from the same cells and analysed for the expression of DEC1.

were cultured in the presence or absence of tetracycline. After a 48 h incubation, the proliferation rate was determined. As shown in Figure 4(A), the sense, but not the antisense or mutant, line exhibited a significant proliferation inhibition. Such an inhibitory effect correlated well with the amount of serum supplemented in the media (Figure 4B). The serum-related inhibition was more evident in cells seeded at higher densities (results not shown). Furthermore, the inhibition activity was correlated well with the abundance of induced DEC1 (Figure 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. To test whether DEC1 was anti-apoptotic, DEC1-stable transfectants were cultured in serum-free media. After a 48 h incubation, the morphological changes were studied microscopically. When cultured in the presence of tetracycline, the sense line displayed the appearance of normal cells (Figure 5A, upper panel). However, the same line, when cultured without tetracycline, rapidly underwent apoptotic changes including condensed nuclei, blebbing of plasma membrane, decreased cell size and formation of aggregates (Figure 5A, upper panel). In contrast, the mutant line underwent apoptotic changes whether or not tetracycline was added to the media (Figure 5A, middle panel). Similar to the mutant line, the antisense line underwent a cell-dying process (Figure 5A, lower panel). In contrast with the apoptotic appearance seen for the mutant line, the changes of the antisense line were associated by excess granule deposit, enlarged cell body, shrunk cell processes and lack of a 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 5A).

To link the morphological changes to DNA fragmentation, another hallmark for cell apoptosis, stable transfectants were subjected to serum deprivation in the presence or absence of tetracycline. After a 48 h incubation, soluble DNA was isolated and analysed by agarose-gel electrophoresis. As shown in Figure 5(B), 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 5B).

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 an apoptotic process [30]. 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 6(A), when cultured in the presence of tetracycline, the sense line showed a marked decrease in the activity of caspases 3/7 and 9 but not caspase 8. The decrease in caspase 3/7 activity was more profound than that in 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 in the caspase activity whether or not tetracycline was added [results shown in Figure 6(B) only for caspase 3/7].

Although caspases show a preference for peptidyl substrates, they are not very specific. To establish further the selectivity of

Figure 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 (Tet, 0–1 µg/ml) overnight and then subjected to serum deprivation for 48 h. 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 the activity 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 caspases 3, 7, 8 and 9. Lysates (50 µg) from the stable transfected sense line cultured in the absence or presence of tetracycline (1 µg/ml) during serum starvation were analysed by Western blotting with antibodies specific to cleaved procaspase 3, 7, 8 or 9. The blots were developed by chemiluminescent substrate.

DEC1 in the activation of several procaspases, immunoblotting analyses were performed with antibodies specific to cleaved procaspase 3, 7, 8 or 9. As shown in Figure 6(C), when cultured

in the presence of tetracycline, the sense line showed a marked decrease in the levels of cleaved procaspases 3, 7 and 9. In contrast, little changes were detected in the levels of cleaved procaspase 8. These results are consistent with the data from enzymic assays (Figure 6A) and provide further evidence that DEC1 selectively suppresses the activation of procaspases. The selective suppression in the activation of procaspases 3, 7 and 9 over procaspase 8 suggests that DEC1-mediated anti-apoptosis 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 (results not shown).

DISCUSSION

Human DEC proteins, along with mouse STRA and rat SHARP, represent a new class of bHLH transcription factors [11–14]. This report describes the functional characterization of DEC1. Subtractive experiments with matched samples, followed by Northern- and Western-blot analyses, demonstrate that DEC1 is highly expressed in the tumour, 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-apoptotic effects distinguish DEC1 from other bHLH proteins, particularly those that have oncogenic significance. The Myc oncoproteins exhibit intrinsically growth-promoting activity [31,32]. Experimentally enforced *c-myc* expression inhibits differentiation and induces cell proliferation. Instead of suppressing apoptosis, overexpression of Myc proteins induces it [33]. The TAL1 protein, while absent in normal adult T lymphocytes, is constitutively expressed in more than 60% of T-cell acute lymphoblastic leukaemia. In contrast with DEC1, ectopic expression of TAL1 promotes cell proliferation and facilitates p53-mediated apoptosis [34]. Twist, another bHLH protein involved in developing oncogenic phenotypes, promotes both proliferation and survival [35]. ID proteins, lacking the DNA-binding domain, heterodimerize with many other bHLH proteins, and therefore act as dominant-negative regulators and have a broad spectrum of biological activities [36]. Similar to Myc protein, ID proteins are proliferation-promoting and pro-apoptotic [36–38].

The proliferation inhibition and anti-apoptosis by DEC1 are likely to be active events through direct DNA binding. In the present study, we demonstrate that only the sense line but not the mutant line showed proliferation inhibition and anti-apoptotic 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 [24]. In addition, the mutant line was shown to repress E47-mediated transcription activity towards an E-box reporter in a tetracycline-dependent manner (results 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 [11]. The repressive activities of STRA13 are

achieved by recruiting co-repressor histone deacetylase (HDAC1) or interacting with basal transcription factor TFIIB [21]. 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 towards any known consensus N- or E-box sequences [11], whereas a glutathione S-transferase fusion protein containing the N-terminal 122 residues of DEC1 is recently reported to bind to E-box and related sequences [25].

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 in the levels of DEC1 were detected (Figure 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 phase. Only the first two factors were shown to induce DEC1 drastically [24,39] (Figure 2B). Generally, a sustained induction by cAMP, hydroxyurea and nocodazole was observed as early as 6 h after treatment [39] (Figure 2). Cycle synchronization normally takes approx. 24 h. 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 a suppressor on cell proliferation (Figure 4). It should be noted that induction of DEC1 probably provides a cytoprotective role as confluent cells usually experience nutrient depletion and cell-cycle blockers are generally cytotoxic.

Anti-apoptotic 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 the onset of expression as early as the end of embryonic development (E20), suggesting that DEC/STRA/SHARPs play a physiological role [11–14]. In the present study, 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 anti-apoptotic action against serum deprivation is particularly relevant to the survival of tumours. Many cancer tissues, particularly solid tumours, are poorly vascularized, which resembles the condition of serum starvation [40–42]. Poor vascularization leads to hypoxia, and survival of hypoxia is an important mechanism of tumour expansion. Interestingly, hypoxia drastically induces the expression of DEC1 and the induction is abolished by von Hippel–Lindau tumour-suppressor protein [24], a factor that regulates activity of the hypoxia-inducible factor 1 (HIF-1). The HIF-1-deficient tumours, although showing reduced vascularization, exhibit a higher expansion rate than HIF-1-expressing tumours [40–42]. However, it remains to be established whether HIF-1-deficient tumours express high levels of DEC1 and whether DEC1 expression is a contributing factor to the faster expansion of HIF-1-deficient tumours.

It is interesting to notice that the stable transfectant expressing antisense DEC1 was morphologically different from the mutant line upon serum starvation (Figure 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 5), suggesting that these cells undergo apoptosis to a comparable extent. The morphological difference, however, was

likely due to the difference in 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 probably suppressed. We tested several lines for each type of stably transfected cells and the same results were consistently obtained, excluding the possibility that the difference in the location of the transgene inserted into 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 anti-apoptotic 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 the present study functionally distinguish them from other bHLH proteins as well.

This work was partially supported by the National Institute of General Medical Sciences grant GM61988.

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Received 2 April 2002/10 June 2002; accepted 15 July 2002

Published as BJ Immediate Publication 15 July 2002, DOI 10.1042/BJ20020514