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Caspase-1 Is a Direct Target Gene of ETS1 and Plays a Role in ETS1-Induced Apoptosis

Huiping Pei, Chunyang Li, Yair Adereth, Tien Hsu, Dennis K. Watson, and Runzhao Li

Department of Pathology and Laboratory Medicine, Hollings Cancer Center, Medical University of South Carolina, Charleston, South Carolina

Abstract

ETS1, the founding member of Ets transcriptional factor family, plays an important role in cell proliferation, differentiation, lymphoid cell development, transformation, angiogenesis, and apoptosis. Previous work has shown that ETS1 represses tumorigenicity of colon carcinoma cells *in vivo*, and that the p42-ETS1 protein bypasses a defect in apoptosis in colon carcinoma cells through the up-regulation of caspase-1 expression. In this report, we show that expression of p42-ETS1 inhibits tumorigenicity of colon cancer DLD-1 cells through induction of apoptosis *in vivo*. In support of the hypothesis that caspase-1 might be a target involved in the sensitization of DLD-1 cells to Fas-induced apoptosis by ETS1, overexpression of caspase-1 bypasses Fas-induced apoptosis in these cells as well. Furthermore, ETS1-mediated apoptosis was observed in MOP8 cells, a transformed mouse NIH3T3 cell line. To determine whether ETS1 activates the transcription of caspase-1, luciferase reporters driven by the wild-type and mutant caspase-1 promoters were generated. Both p51-ETS1 and p42-ETS1 transactivated the caspase-1 transcription and a functional Ets binding site is identified in the caspase-1 promoter. Wild-type caspase-1 promoter (pGL3-ICE) was strongly transactivated by ETS1 and this transactivation was dramatically diminished by the mutation of the potential Ets binding site (–525 bp). In addition, electrophoretic mobility shift assay and chromatin immunoprecipitation assay showed complex formation between this binding site and ETS1 proteins. Taken together, ETS1 transcriptionally induces the expression of caspase-1; as such, the regulatory control of caspase-1 expression by ETS1 may underlie the apoptotic susceptibility modulated by ETS1 in specific tumor cells.

Introduction

Apoptosis, or programmed cell death, is a process essential for normal development and homeostasis in multicellular organisms (1). It provides a defense against cell immortalization caused by oncogenesis or viral infection (2–4). ETS1, the founding member of the Ets transcription factor family, is the cellular counterpart of the *v-ets* oncogene of the avian erythroblastosis virus E26 and it plays an important role in cell proliferation, differentiation, lymphoid cell development, transformation, angiogenesis, and apoptosis (5,6).

ETS1 encodes two proteins, a 51 kDa protein (p51-ETS1) and a 42 kDa protein (p42-ETS1), encoded by an alternately spliced mRNA lacking exon 7 (7). A correlation between ETS1 expression and tumor invasiveness and metastases is emerging based on clinical and experimental observations: ETS1 expression is induced in many invasive cancers and many of the ETS1 target genes are involved in the control of tumor metastases (5,6). However, the

Requests for reprints: Runzhao Li, Hollings Cancer Center, Medical University of South Carolina, Room 315, 86 Jonathan Lucas Street, Charleston, SC 29425. Phone: 843-792-2443; Fax: 843-792-5002; E-mail: lir@musc.edu.

Note: This study is dedicated to the memory of Dr. Takis S. Papas, a pioneer of molecular oncology, on the fifth anniversary of his death, Nov 19, 2004.

role of ETS1 in the regulation of cell proliferation and death remains enigmatic. ETS1-deficient mice show that the ETS1 proto-oncogene is required for normal survival and activation of B cells, T cells (8,9), and natural killer T cells (10). ETS1 protects vascular smooth muscle cells from undergoing apoptosis by activating the transcription of p21^{waf1/cip1} (11). In contrast, ETS1 plays a role of proapoptosis in endothelial cells (12) and embryonic stem cells (13). Previous work has shown that ectopic expression of either p51-ETS1 or p42-ETS1 in ETS1-null colon carcinoma DLD-1 cells reduces tumorigenicity, and this activity requires ETS1 transcriptional activity (14,15). Interestingly, DLD-1 cells undergo apoptosis in either low-serum or Fas antibody treatment following expression of p42-ETS1, but not p51-ETS1 (15, 16). However, direct target genes of ETS1 that contribute to apoptosis are not fully understood.

We have previously shown that caspase-1 may be an ETS1 downstream target gene associated with the modulation of the susceptibility of cancer cells to programmed cell death (16). Caspase-1 plays a prominent role in inflammatory responses (17,18). Because a typical apoptotic cascade remains intact in caspase-1^{-/-} mice (17,18), caspase-1 is not necessary in normal development. However, caspase-1 plays a role in the apoptotic induction of inflammatory response cells (19–22), transformed fibroblast cells (23), tumor cells (24–27), and especially of cells under stresses such as Fas cross-linking (28,29), granzyme B stimulus (30), and growth factor deprivation (31). The up-regulation of caspase-1 by another Ets family member, PU.1, also occurs in erythroleukemia cells undergoing apoptosis (32,33). Therefore, the determination of whether caspase-1 is a direct target of ETS1 is substantial for understanding the apoptotic control of cancer cells.

In this report, we show that p42-ETS1 inhibits tumorigenicity of colon cancer DLD-1 cells by inducing apoptosis *in vivo* using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays. The up-regulation of caspase-1 by ETS1 was validated by immunohistochemical and Northern blot analyses. Overexpression of caspase-1 in DLD-1 colon cancer cells sensitizes these cells to Fas-induced apoptosis, suggesting a role of caspase-1 in ETS1-mediated apoptosis. Interestingly, we also observed that both p42-ETS1 and p51-ETS1 proteins induced apoptotic death in MOP8 cells, an SV40-polyoma transformed mouse NIH3T3 cell line. To determine whether caspase-1 is a direct target of ETS1, luciferase reporters driven by either wild-type or mutant caspase-1 promoters were generated. Our results showed that ETS1 transactivates caspase-1 transcription, and identified a functional ETS1 activation site in the caspase-1 promoter.

Materials and Methods

Xenograft tumor model in nude mice

DLD-1 stable transfectants containing either p42-ETS1 Tet-off vector (Δ VII-19) or control vector (tTA1) were maintained in the presence of tetracycline (2 μ g/mL). Following harvest and resuspension in 100 μ L of PBS, 2×10^6 cells of each clone were injected s.c. into the right anterior flank of 6- to 8-week-old athymic nude mice (Harlan, Indianapolis, IN). Tumors were measured once weekly with calipers and tumor volumes were calculated using the formula $[0.52 (L \times S^2)]$, where L represents the largest tumor diameter and S represents the smallest tumor diameter.

Tet-off system and cell viability assays

MOP8 cells (ATCC no. CRL1709), SV40-polyoma transformed mouse NIH3T3 cells, were transfected with the indicated expression vector, and stable transfectants expressing p42-ETS1, p51-ETS1, and a mutant p42-ETS1 were isolated. Clones were maintained in RPMI 1640 with 15% fetal bovine serum in the presence of G418 (400 μ g/mL), tetracycline (2 μ g/mL), and hygromycin (100 μ g/mL). Cell viability was carried out using 3-(4,5-dimethylthiazol-2-

yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) assays as previously described (16).

Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling assays

The assay was done as described by the manufacturer (Roche, Indianapolis, IN).

Cloning of caspase-1 promoter and generating the point mutations by site-directed mutagenesis

Human caspase-1 promoter DNA was amplified by Pfu PCR from human genomic DNA (Clontech, Palo Alto, CA) based on published sequence (1,480 bp; accession no. L27475) by using forward primer ACCATCGGAGCTCATGAGACATTCAT and reverse primer TGGTCGACTGAACTGAAAGTATGCT. pTOPO-ICEp was generated by inserting this PCR fragment with *KpnI/EcoRV* sites into a TOPO vector (Roche) with *KpnI*/blunted *SmaI* sites. Then, pTOPO-ICEp fragment with *EcoRV/BamHI* sites was inserted into a luciferase reporter vector (pGL3 enhancer, Promega, Madison, WI) at *BglIII/SmaI* sites, generating pGL3en-ICEp. Two mutation constructs were generated using the Excite PCR-Based Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA); one is pGL3en-ICEpD, which has a large deletion between -936 and -655 bp, and the other is pGL3en-ICEpM3, which has point mutations in EBiceII site (TTCCGGC to TTCTAT). All constructs were confirmed by sequencing. Caspase-1-expressing vector (pBabe-Gal-ICE) was kindly provided by Dr. J.Y. Yuan. The control vector (pBabe-Gal) was generated by deleting the caspase-1 coding fragment by *BamHI/EcoRI* digestions.

Cell lines, transfections, and transient reporter assays

Transient transfections were carried out in the human colon carcinoma cell line DLD-1 (ATCC no. CCL-221) and COS-1 (ATCC no. CRL-1650) cells using FuGENE 6 (Qiagen, Valencia, CA) as per directions of the manufacturer. Reporter assays were carried out as previously described (34). A luciferase reporter plasmid (e.g., thymidine kinase promoter-Renilla) was used as an internal control for transfection efficiency. During the investigation of ETS1 transactivation, we found that ETS1 also affected the luciferase activity of the control thymidine kinase promoter. Therefore, we used Western blotting for controlling ETS1 expression levels. Equivalent amounts of cell protein extract were used for reporter assays and Western blots. All transfections were done at least thrice in duplicate or triplicate.

Apoptosis assays

The Fas monoclonal antibody CH-11 (MBL, Nagoya, Japan) was used for inducing apoptosis. DLD-1 cells were plated onto six-well dishes the day before transfection at 2×10^5 cells/well and transfected with FuGENE 6 (Roche). 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) staining was done for 4 hours. The percentage of apoptotic cells was determined by the number of blue cells with apoptotic morphology divided by the total number of blue cells. At least 500 cells from six random fields were counted in each experiment, and the data shown are the average and SD of at least three independent experiments.

Western blots and Northern blots

Western blots were developed by enhanced chemiluminescence. Anti-ETS1 (C-20) was purchased from Santa Cruz (Santa Cruz, CA), anti- β -actin (AC-15) was from Sigma, and anti-caspase-1 was from Biosource International (Camarillo, CA). Total RNA was extracted from 10^7 cells using Trizol (Invitrogen, Carlsbad, CA), and 10 μ g of denatured RNA were separated in a 1% agarose gel containing 2% formaldehyde, blotted on a nylon membrane (Hybond-N; Amersham), UV cross-linked, and hybridized in Quik-Hyb (Stratagene) using random-primed labeled caspase-1 and S26 cDNA probes.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assay (EMSA) was done as follows. The known ETS1 consensus sequence oligonucleotide ETS1-3 (GATCTCGAGCCGGAAGTTCTGA; ref. 35) and the potential ETS1 binding oligos, EBiceI (ATAAACCTCCAGGATGGGTGGGTG) and EBiceII (AATAACTGCATTCCGGCCTGCACGACGACA), and their complements were synthesized, prepared in double-stranded form, and end labeled with [α - 32 P]dCTP using Klenow enzyme. DNA (10 to 20 fmol) was mixed with 5 μ L of nuclear extracts (5 μ g of protein), 1.2 μ g of poly(deoxyinosinic-deoxycytidylic acid), and 2 μ L of 10 $\times$ reaction buffer (100 mmol/L Tris-HCl, 25 mmol/L KCl, 10 mmol/L EDTA, 50 mmol/L DTT, 0.5% NP40, and 10 mg/mL bovine serum albumin). Water was added to each reaction to bring to a final volume of 20 μ L and the mixture was incubated for 30 minutes at room temperature. Reaction products were separated by 4% PAGE for 3 hours in 0.25 $\times$ Tris-borate-EDTA running buffer at 200 V and visualized by autoradiography.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation assays were done as previously described (36) with modifications. Briefly, cells (10^7) grown on a 10-cm plate to 85% confluence were cross-linked with 1% formaldehyde for 15 minutes at 37°C. The fixed cells were washed twice in cold 1 $\times$ PBS and lysed in 1 mL of chromatin immunoprecipitation cell lysis buffer [5 mmol/L PIPES (pH 8.0), 85 mmol/L KCl, 0.5% NP40, 25 μ L protease inhibitor mixture] at room temperature for 10 minutes. Nuclei were collected by centrifugation at 1,000 $\times$ g at 4°C and lysed in chromatin immunoprecipitation nuclei lysis buffer [1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.1), 50 μ L of protease inhibitor mixture]. DNA was sheared by sonication to yield an average fragment size of 500 bp. After centrifugation, the supernatant was diluted in chromatin immunoprecipitation dilution buffer, precleared, and then incubated overnight at 4°C with or without anti-ETS1 (C-20) polyclonal antibody. Immune complexes were recovered by the addition of 80 μ L of salmon sperm DNA/protein A-agarose-50% slurry and incubation for 2 hours at 4°C with rotation. Agarose beads were pelleted by gentle centrifugation (1,000 rpm). The beads were sequentially washed with low-salt and high-salt buffer, LiCl buffer, and finally twice with Tris-EDTA buffer. After washing, the immune complexes were eluted by incubation for 15 minutes at 25°C with 500 μ L of fresh elution buffer (1% SDS, 0.1 mol/L NaHCO₃). To reverse the cross-linking of DNA, 20 μ L of 5 mol/L NaCl were added and incubated for 4 hours at 65°C. After treatment with proteinase K for 1 hour at 45°C, DNA was purified using the QIAquick PCR Purification Kit (Qiagen). The DNA pellets were resuspended in 50 μ L of Tris-EDTA buffer and subjected to PCR amplification. Primers used to amplify genomic DNA were as follows: EBSiceI, 5' CGACATTCTCATTCCAGAGCCTATG, 3'GACAGGGTCTCCTTGTGTTTCCTA; EBSiceII, 5'GAGGCTGAGTTGGAAGAATCATCTG, 3' GTTGTGGGTAATGTATGTCCCTGTG; and β -actin, 5' TACCTACACCCACAACACTGTCTTAG, 3'AATCTGGCACCCACCTTCTACAA. DNA gels were stained with SYBR green (Cambrex, Baltimore, MD).

Results

Ectopic expression of p42-ETS1 inhibits tumorigenicity of colon cancer DLD-1 cells through apoptosis *in vivo*

Previous work has indicated that ETS1 represses tumorigenicity of DLD-1 in nude mice (14) and induces DLD-1 cell apoptosis in low-serum condition (15) or following Fas antibody cross-linking *in vitro* (16). From those observations, we speculated that the loss of tumorigenicity of the DLD-1 cells expressing ETS1 *in vivo* must have undergone apoptosis or have restricted proliferation. To examine the contribution of apoptosis *in vivo*, we used a xenograft tumor model of DLD-1 colon cancer cells with inducible p42-ETS1 expression. The tumorigenicities

of p42-ETS1–expressing cells and tTA1 vector control cells were compared and their apoptotic features were determined *in vivo* by TUNEL assays. Consistent with previous observations, tumor formation was strongly inhibited in p42-ETS1 transfectants: when tumor sizes of DLD-1 transfectants expressing p42-ETS1 became palpable (average volume, 0.3 cm³) at 7th week, the tumors from the control vector transfectant (tTA1) had developed into solid tumors with an average volume of 4 cm³ (Fig. 1A). However, it is unknown whether observed inhibition of tumorigenicity by p42-ETS1 *in vivo* was associated with apoptosis. For detecting DNA strand breaks, one of the hallmarks of programmed cell death, TUNEL assays were used at the end of the experiment when tumor volume reached a minimum of 0.2 cm³ in p42-ETS1 transfectants. By using fluorescence microscopy, tumor sections derived from p42-ETS1–expressing cells exhibited extensive positive staining, which represents apoptosis, whereas tissue sections from the control group were not significantly stained (Fig. 1B, *c* and *d*). H&E staining of the same section showed that these positive signals were localized in areas within the xenograft tumor tissues (Fig. 1B, *a* and *b*). To further show that ETS1 induces caspase-1 expression *in vivo*, immunohistochemical study of caspase-1 was also carried out in these tissue sections. Increased caspase-1 protein expression was observed in the tumor tissues from p42-ETS1 (Δ VII-19), but not in the control empty vector (tTA1; Fig. 1B, *e* and *f*). Consistent with these observations, the expression of caspase-1 RNA was significantly up-regulated in p42-ETS1–expressing cells. Although p42-ETS1 and p51-ETS1 protein levels were readily induced at equivalent levels (Fig. 1C, *bottom*), the level of p51-ETS1 increased expression of caspase-1 is significantly less than that observed with p42-ETS1 (Fig. 1C).

ETS1 induces apoptosis in transformed fibroblast cell MOP8

Although both p51-ETS1 and p42-ETS1 repressed tumorigenicity of the DLD-1 cells *in vivo* (14,15), only DLD-1 cells with expression of p42-ETS1, but not p51-ETS1, underwent apoptosis following either low-serum (15) or Fas antibody treatment (16). To further characterize the modulation of apoptosis by ETS1, we examined whether p42-ETS1 and p51-ETS1 affect apoptosis in cells other than those derived from epithelial cancers. MOP8, a SV40-polyoma transformed mouse NIH3T3 cell line, has a closer setting with tumor cell than its parental NIH3T3 cell and it has no detectable ETS1 expression. Therefore, MOP8 was transfected with Tet-off constructs of p42-ETS1, p51-ETS1, or the control vector tTA1, and stable clones were selected (Materials and Methods). ETS1 protein expression was induced after tetracycline withdrawal (Fig. 2A). Compared with either the cells containing the control vector (M10-3) or individual cell clones grown in the presence of tetracycline, p42-ETS1 significantly reduced cell viability (Mp42-1, Mp42-8, and Mp42-13) at 96 hours after tetracycline withdrawal. Interestingly, p51-ETS1 also induced cell death with a similar pattern (Mp51-1 and Mp51-14). In addition, it was notable that ETS1 protein levels were correlated with the extent of reduced cell viability (Fig. 2B). MTT assay was used for measuring cell viability because it is a conventional and economical means to quantify large amounts of samples at multiple time points. To further define apoptosis of MOP8 cells after ETS1 induction, *in vitro* TUNEL assays were applied. Positive fluorescence staining can be observed in either p51-ETS1 (Mp51-1)– or p42-ETS1 (Mp42-8)–expressing cells, whereas the control (M10-3) cells were unstained, suggesting that the reduced cell viability measured by the MTT assay was due to a significant extent of apoptotic cell death of MOP8 cells (Fig. 2C). We were unable to detect a significant DNA ladder in MOP8 cells (data not shown); a possible reason for this is that DNA laddering is less sensitive for identifying nonsynchronous and slow-progressing apoptotic process, which is apparently the case of ETS1-induced event (Fig. 2B).

Caspase-1 sensitizes DLD-1 colon cancer cells to Fas-induced apoptosis

Among the genes that might be involved in the modulation of Fas-induced apoptosis, caspase-1 is up-regulated by p42-ETS1, suggesting a potential role of caspase-1 in the apoptotic induction of colon cancer cells (16). Therefore, we speculated that caspase-1 may play a role in Fas-

induced apoptosis in DLD-1 colon cancer cells. To test this, a caspase-1 expression vector and control vector were transiently transfected into DLD-1 cells. In caspase-1-expressing vector (pBabe-Gal-ICE), caspase-1 and β -galactosidase expressions are independently controlled by different promoters; the control vector (pBabe-Gal) expresses only β -galactosidase. Twenty-four hours after transfection, cells were treated with Fas antibody for another 24 hours. Then, the cells were fixed and stained for β -galactosidase. Transfected cells were identified by β -galactosidase activity and the ratios of the dead cells versus the total transfected cells were then calculated. As observed under the light microscope, the cells showed no sign of cell death following Fas antibody treatment whether or not they express the exogenous β -galactosidase (Fig. 3A, pBabe-Gal). However, many of the cells expressing exogenous caspase-1 underwent apoptosis following Fas antibody treatment whereas the untransfected cells in the same group remained viable (Fig. 3A, pBabe-Gal-ICE). Furthermore, with the increase of the Fas antibody concentrations, the cells expressing caspase-1 underwent apoptosis in a dose-dependent manner; the cells expressing control vector had no significant increases of cell death (Fig. 3B). Consistent with the previous observations that extra triggers, either low serum or Fas treatment, are required for apoptotic induction in ETS1-expressing DLD-1 cells (15,16), DLD-1 cells expressing exogenous caspase-1 underwent apoptosis only after Fas treatment. This data is also consistent with our previous observation that caspase-1-specific inhibitor blocks p42-ETS1-induced apoptosis in DLD-1 cells (16), providing strong support for the notion that caspase-1 plays an essential role in ETS1-induced apoptosis in colon cancer cells.

The caspase-1 promoter region contains ETS1 binding site and ETS1 transactivates reporter gene driven by caspase-1 promoter

Thus far, we have shown that both p42-ETS1 and caspase-1 can promote Fas-induced apoptosis in DLD-1 cells. Computer-assisted promoter analysis of the caspase-1 5' region (37) identified two potential Ets binding sites in the promoter region of caspase-1, designated EBSiceI (-776) and EBSiceII (-525; Fig. 4A). To determine whether these sites are functional response elements for ETS1, a 1,450-bp fragment of the caspase-1 5' region was cloned from human genomic DNA by Pfu PCR and placed upstream of the luciferase reporter gene in pGL3 vector to generate pGL3-ICE. p42-ETS1 strongly activated the wild-type caspase-1 promoter driving reporter (pGL3-ICE), with luciferase activities correlating with the ETS1 expression levels (Fig. 4B, lanes 1–5). We previously showed that a dominant negative ETS1 form, which lacks transcriptional activity (14), was unable to facilitate Fas-induced apoptosis in DLD-1 cells (16). Such a construct (p42-mETS1) also failed to transactivate the caspase-1 luciferase construct (Fig. 4B, lane 6), demonstrating that caspase-1 promoter is responsive to ETS1, providing a potential mechanism that correlates ETS1 transcriptional activity with apoptosis modulation. Furthermore, the reporter assays showed that p51-ETS1 also strongly activated the caspase-1 promoter reporter (more than 14-fold) with increased expression levels of p51-ETS1 protein (Fig. 4B, lanes 7–11). These results show that both p42-ETS1 and p51-ETS1 are able to transactivate the caspase-1 reporter gene in COS-1 cells.

To determine the specificity of the potential Ets binding sites for the ETS1 transactivation function, we used a point mutation, pGL3/ICEM, which lacks the EBSiceII site (-525), and a deletion mutation, pGL3/ICED, which lacks both EBSiceI (-776) and EBSiceII (-525) sites. As shown in Fig. 5, p42-ETS1 activates the reporter driven by the wild-type caspase-1 promoter. However, mutation of Ets core binding sequence of EBSiceII (-525; **TTCCGGC** to **TTCTAT**) reduces the activity of p42-ETS1 in pGL3/ICEM reporter to less than one fourth of that in the wild-type reporter (Fig. 5). Deletion of both potential Ets binding sites (pGL3/ICED) further reduces the activity of p42-ETS1 to around 5% of its activity in the wild-type reporter. The p42-ETS1 protein levels were monitored with the same cell extracts used for the luciferase reporter assays. For comparison among the different reporter constructs, pGL3 empty vector was used as a control to normalize the system (Fig. 5, lanes 1, 6, and 11). In

addition, p51-ETS1 was also used for these experiments and similar results were obtained (data not shown). These results strongly suggest that EBSiceI and EBSiceII in caspase-1 promoter are functional Ets binding sites and may bind to ETS1 protein.

ETS1 specifically binds the potential Ets binding site

Although the caspase-1 promoter is functionally modulated by ETS1 protein, the evidence of the functional Ets binding site(s) (e.g., protein-DNA complex) will be necessary for determining whether caspase-1 is a direct downstream target of ETS1. It is possible that ETS1 induces caspase-1 by interacting or tethering with other transcription factors because the combinatorial control is a characteristic property of ETS1 (38). To this end, oligonucleotides corresponding to the potential Ets binding sites EBSiceI and EBSiceII (Fig. 6) were synthesized and used in EMSAs. An oligonucleotide (ETS1-3) containing the ETS1 consensus sequence (35) was used as a positive control. Both *in vitro* translated p51-ETS1 and p42-ETS1 bound to the control oligonucleotide, and after addition of an ETS1 monoclonal antibody (E44), p42-ETS1 complex was supershifted (Fig. 6, lane 6, *asterisk*) whereas p51-ETS1 complex became a smear (Fig. 6, lane 5). The same phenomena have been noted (39). EBSiceII was also able to form a complex with both p51-ETS1 and p42-ETS1. However, after addition of antibody E44, the supershift band can be seen only in the p42-ETS1-containing complex (Fig. 6, lane 12). The lack of the supershift in p51-ETS1-containing complex may result from altered binding kinetics of ETS1 variant forms (40). Furthermore, we were unable to detect the complex formation between EBSiceI probe and ETS1 under similar conditions (data not shown). The EMSA assays were carried out further with nuclear extracts from cells transiently transfected with p51-ETS1, p42-ETS1, and the control vector SG5, respectively. Consistent with the results obtained with the *in vitro* translated proteins, both p51-ETS1 and p42-ETS1 formed protein-DNA complexes with EBSiceII (Fig. 7A, lanes 8 and 9). Both p42-ETS1 and p51-ETS1 complexes were greatly reduced when the monoclonal anti-ETS1 antibody (E44) was added (Fig. 7A, lanes 11 and 12, *open circles*). However, only p42-ETS1 complex was supershifted (Fig. 7A, lane 12) whereas p51-ETS1 complex became a smear (Fig. 7A, lane 11). ETS1-3 was used as a positive control in the EMSA (Fig. 7A, lanes 1–6). To further verify the specificity of the ETS1:DNA complex formation, increasing amounts of unlabeled probe ETS1-3 (containing Ets binding site) and its mutant form mETS1-3 (containing mutant Ets binding site) were used as competitors in the EMSA assays. The p42-ETS1/EBSiceII complex was competitively blocked by the ETS1-3 probe, whereas the mutant EBS1-3 probe (mETS1-3) did not compete (Fig. 7B, lanes 3–5 and 6–8). The supershift of this specific DNA:protein complex by anti-ETS1 antibody further showed that the DNA-protein complex is ETS1 specific (Fig. 7B, lane 2). These results clearly show that EBSiceII is one of the functional Ets binding sites in the caspase-1 promoter region.

To show direct binding of ETS1 to the endogenous caspase-1 promoter *in vivo*, we did chromatin immunoprecipitation assay using chromatin prepared from DLD-1 stable transfectants containing either control vector (tTA1), p51-ETS1 (E1-10), or p42-ETS1 Tet-off vector (Δ VII-19). ETS1 antibody C20 has been shown to be suitable for chromatin immunoprecipitation analyses (36,41). We detected ETS1 binding on the EBSiceII region (–608 to 408) of caspase-1 promoter in cells expressing p42-ETS1 (Δ VII-19), but not in cells expressing either p51-ETS1 (E1-10) or the control vector (tTA1; Fig. 7C). Moreover, the EBSiceI region (–881 to 696) of caspase-1 promoter was not detected in immunoprecipitated complexes from any DLD-1 transfectants. The negative control group had no antibody and did not immunoprecipitate either genomic region of the caspase-1 promoter in cells expressing p42-ETS1 (Fig. 7C, *No-ab*, lane 2), confirming the specificity of this assay. These results extended the EMSA observations demonstrating that ETS1 binds to EBSiceII of caspase-1 promoter *in vitro* and *in vivo*.

Discussion

Our previous work (16) and this report show that ETS1 protein can be a potent inhibitor of the uncontrolled growth of cancer cells and transformed cells by apoptotic induction. Furthermore, we identified a functional ETS1 binding site in the caspase-1 promoter and showed that ETS1 directly activates transcription of the caspase-1 gene.

Oncoproteins of Ras, myc, Src, and nuclear factor κ B are prominent during transformation and tumorigenesis, and they also carry the ability to trigger apoptosis and may thus suppress transformation or render cells susceptible to apoptosis (42,43). Similar to the role of apoptosis modulation by these oncoproteins, ETS1-mediated apoptosis induction is also cell context specific, which might be related to genetic alternation, specific signaling transduction pathways, and interacting protein partners. First, although ETS1 plays a proapoptotic role in endothelial cells (12), DLD-1 cells (15), and MOP8 cells (this report), it protects vascular smooth muscle cells from undergoing apoptosis (11). Thus, apoptotic modulation by ETS1 is specific for certain cell types. Second, as an end-effector of signal transduction pathways, ETS1 transduces environmental signals into transcriptional control of apoptosis-related target genes. Consistently, ETS1-mediated apoptosis is affected by stimuli or stresses. ETS1-mediated apoptosis may require an additional trigger such as low serum (15), Fas cross-linking (16), or UV irradiation (13). We have observed that both p42-ETS1 and p51-ETS1 are able to transactivate the caspase-1 promoter (Fig. 4B). However, we also observed that p42-ETS1 up-regulates the expression of endogenous caspase-1 transcription to a much higher level than p51-ETS1 in DLD-1 cells (Fig. 1C), indicating that different forms of ETS1 may be modulated by specific interacting partner(s). It is known that p53 transcriptionally induces caspase-1-dependent apoptosis (44,45) and that p53 and ETS1 act synergically in the combinatorial transcriptional control of proapoptosis genes such as bax and cytochrome *c* (13,38). We have previously shown that Ets1 associated protein 1/death-associated protein 6 (Daxx) cooperates with ETS1 to suppress the expression of the antiapoptotic protein bcl2 (34). Interestingly, Daxx is known as an important component of the apoptosis-related suborganelle nuclear bodies (or promyelocytic leukemia oncogenic domains or promyelocytic leukemia nuclear bodies; refs. 46,47). The status of these and other interacting partners might be critical for the role of ETS1 in apoptosis induction (5,38).

As a direct target of ETS1, caspase-1 is likely to be important for ETS1-mediated apoptosis. Caspase-1 expression is negatively regulated in epithelial cancer cells (48) and lower levels of caspase-1 are observed in advanced tumors compared with early-stage tumors (49,50), suggesting that caspase-1 plays an important role in regulating the apoptotic process of these cancer cells. The enhanced apoptotic induction by expressing exogenous caspase-1 in DLD-1 cells (Fig. 3) supports this hypothesis. Therefore, caspase-1 is one of the downstream genes that contribute to the ETS1-mediated apoptosis.

The unexpected result that ETS1-induced apoptosis in MOP8 does not require an additional trigger suggests that these cells are sensitive to apoptotic induction. Although the mechanism by which ETS1 mediates apoptosis is not fully understood, the fact that both p51-ETS1 and p42-ETS1 proteins transactivate caspase-1 transcription in the reporter assays supports previous reports that both p51-ETS1 and p42-ETS1 repress tumorigenicity in DLD-1 cells (14,15). In DLD-1 cells, the level of caspase-1 mRNA induced by p42-ETS1 expression is significantly greater than that observed following p51-ETS1 expression (Fig. 1). The lower transcriptional activation of caspase-1 by p51-ETS1 in DLD-1 cells reflects that (a) proteins that interact with exon 7 could interfere with interactions between ETS1 and its coactivators; (b) other regulatory elements outside the fragment (–1,450 bp) used in the reporter assays might also be involved in the ETS1-mediated caspase-1 transcriptional control; or (c) the potential binding sites for other transcription factors including p53 might be involved. Although we did

not observe the complex formation between EBSiceI and ETS1 protein by using EMSA or chromatin immunoprecipitation assay, the promoter containing the mutated EBSiceII site retains 25% activity (Fig. 5), suggesting that EBSiceI may be a weak site for ETS1 protein: the ETS1-EBSiceI complex formation may be dependent on specific cofactors or may depend on the native conformation of specific proteins (e.g., functional binding sites of the transcription factors signal transducers and activators of transcription 1 and p53 have been identified in the promoter region of caspase-1; refs. 45,51).

In summary, caspase-1 is a direct target of ETS1 and apoptotic modulation by ETS1 is dependent on specific cell context. Further characterization of ETS1-interacting partners may increase understanding of the mechanisms that modulate ETS1-mediated tumor cell death versus survival.

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Figure 1.

DLD-1 colon carcinoma expressing p42-ETS1 undergoes apoptosis *in vivo*. **A**, DLD-1 stable transfectants containing either p42-ETS1 Tet-off vector (Δ VII-19) or control vector (tTA1) were maintained in the presence of tetracycline (2 μ g/mL). Of each clone, 2×10^6 cells/100 μ L PBS were injected s.c. into the right anterior flank of 6- to 8-week-old athymic nude mice (eight mice in each group). Tumor volumes were measured and recorded once a week for 7 weeks. **B**, tissue sections from the above mice were used for H&E staining ($\times 100$), TUNEL assay (fluorescence microscopic images, $\times 100$), and immunohistochemical detection of caspase-1 ($\times 200$). Endogenous caspase-1 was immunostained with anti-caspase-1 (Biosource International). **C**, p42-ETS1 up-regulates endogenous caspase-1 mRNA in DLD-1 cells. Northern blot using total RNA purified from DLD-1 stable transfectants expressing empty vector (tTA1), p42-ETS1 (Δ VII-19), or p51-ETS1 (E1-10), 48 hours after tetracycline withdrawal. Caspase-1 and S26 cDNA fragments were used to probe the corresponding mRNAs and a monoclonal anti-ETS1 antibody (E44) was used to detect the levels of ETS1 proteins.

Figure 2.

Overexpression of p51-ETS1 and p42-ETS1 induces apoptosis in MOP8. **A**, inducible p51-ETS1 and p42-ETS1 expression levels in stably transfected MOP-8 cells. MOP8 cells were either transfected with control vector (clone M10-3), p42-ETS1 (clones Mp42-1, Mp42-8, and Mp42-13), or p51-ETS1 (clones Mp51-1 and Mp51-14) and stable clones were selected. These cell lines were maintained in the presence of tetracycline (2 μ g/mL). The cell lysates from each clone were extracted 48 hours after tetracycline withdrawal and the same amount of protein was electrophoresed and detected by anti-ETS1 (C-20, Santa Cruz) and anti- β -actin (AC-15, Sigma) antibodies. **B**, cells were placed in a 96-well plate and incubated in the presence or absence of tetracycline (2 μ g/mL; +Tc or -Tc). Cell viability was measured by MTT assay (Materials and Methods) at the indicated time points. **C**, representative fluorescence microscopic images of the TUNEL assays show that expression of either p51-ETS1 (Mp51-1) or p42-ETS1 (Mp42-8) induces apoptosis in MOP8 cells 72 hours after tetracycline withdrawal, whereas the control (M10-3) has few apoptotic cells.

Figure 3.

Caspase-1 sensitizes DLD-1 colon cancer cells to Fas-induced apoptosis. *A*, normal and apoptotic DLD-1 cells. DLD-1 cells were transiently transfected with pBabe-Gal vector or pBabe-Gal-ICE. Twenty-four hours after transfection, the cells were treated with anti-Fas antibody CH-11, stained with X-Gal 24 hours after the treatment, and examined by light microscopy. *Arrows*, apoptotic cells. *B*, caspase-1 sensitizes DLD-1 cells to Fas-induced apoptosis. The cells were stained with X-Gal 24 hours after CH-11 treatment and counted for the dead cells among the X-gal positive cells. At least 500 X-Gal staining cells were counted for each point and these results are representative of two independent experiments.

Figure 4.

Caspase-1 promoter region contains functional ETS1 binding sequence and ETS1 transactivates a reporter gene driven by caspase-1 promoter. **A**, schematic representation of caspase-1/ICE promoter-reporter and its mutant constructs, pGL3/ICE, pGL3/ICEM, and pGL3/ICED. Potential Ets binding sites are indicated as EBSiceI and EBSiceII, and their nucleotide sequences are shown at the bottom. The numbers represent the position relative to the transcriptional start site. **B**, activation of caspase-1/ICE promoter luciferase reporters by ETS1. Caspase-1/ICE promoter construct driving the luciferase reporter (pGL3/ICE) was cotransfected in COS-1 cells with either a control vector SG5 (V) or a mutant p42-ETS1 (M, 1 μg) or with increasing amounts of p42-ETS1 or p51-ETS1 (0.25, 0.5, 1, and 2.0 μg),

respectively. Total amounts of DNA were normalized by the addition of control vector SG5. Cells were harvested after 24 hours and an equivalent amount of protein extract was used for the luciferase assay. The expression levels of ETS1 protein were monitored by Western blot using equivalent amounts of protein (20 μ g), which are shown below the graph.

Figure 5.

Potential Ets binding sites in caspase-1 promoter are necessary for its activation by ETS1. Increasing amounts of p42-ETS1 (0.25, 0.5, 1.0, and 2.0 μ g) were cotransfected in COS-1 cells with luciferase reporters driven by either the wild-type caspase-1 promoter (pGL3/ICE, 0.25 μ g), its point mutation (pGL3/ICEM, 0.25 μ g), or its deletion mutation (pGL3/ICED, 0.25 μ g), respectively. Total amounts of DNA were normalized by the addition of control vector SG5. Cells were harvested 24 hours after transfection and an equivalent amount of protein extract was used for the luciferase assay. The expression levels of ETS1 protein were monitored by Western blot using equivalent amounts of protein (20 μ g), which are shown below the graph.

Figure 6.

ETS1 binds to EBSiceII *in vitro*. *In vitro* translated p51-ETS1, p42-ETS1, or SG5 vector-programmed reticulocyte lysate control was incubated with or without anti-ETS1 antibody (E44). [32 P]dCTP-labeled double-stranded oligonucleotide probe ETS1-3 (containing an ETS1 consensus site) or a potential ETS1 binding oligonucleotide derived from the caspase-1/ICE promoter region (EBSiceII) was added and the incubation was continued for 30 minutes. The mixtures were resolved by PAGE and visualized using autoradiography. *Open arrows*, protein-DNA complex specifically in the presence of either p51-ETS1 or p42-ETS1. *Open circle*, disappearance of p51-ETS1 bound band in the presence of E44 antibody, suggesting a

supershift event (see text also); *asterisks*, supershifted complex in the presence of ETS1-specific antibody.

Figure 7.

ETS1 binds to EBSiceII *in vivo*. **A**, COS-1 cells were transiently transfected with control vector, p51-ETS1, or p42-ETS1 as indicated above. Ten micrograms of each nuclear lysate were incubated without (*None*) or with anti-ETS1 antibody (+*E44*) for 20 minutes. [³²P]dCTP-labeled double-stranded oligonucleotide probe ETS1-3 or EBSiceII was added and the incubation was continued for 30 minutes. *Open arrows*, protein-DNA complex specifically in the presence of ETS1. *Open circles*, disappearance of p51 and p42-ETS1 bands in the presence of E44 antibody; *asterisks*, supershifted complex in the presence of ETS1-specific antibody. **B**, EBSiceII-ETS1 binding is competitively blocked by an oligonucleotide containing an ETS1 consensus site. Ten micrograms of nuclear lysate extracted from COS-1 cells expressing p42-ETS1 were incubated without (*lane 1*) or with anti-ETS1 antibody (*E44*; *lane 2*) or with increasing amounts (2×, 10×, and 50×) of unlabeled probes, either ETS1-3 (*lanes 3–5*) or mutant ETS1-3 (*lanes 6–8*), 20 minutes before the addition of the [³²P]dCTP-labeled EBSiceII probe. *Open arrows*, protein-DNA complex specifically in the presence of p42-ETS1; *asterisks*, supershifted complex in the presence of ETS1-specific antibody. **C**, chromatin immunoprecipitation assays using chromatin prepared from DLD-1 stable transfectants containing either control vector (*tTA1*, *lane 3*), p51-ETS1 (*E1-10*, *lane 4*), or p42-ETS1 Tet-off vector (*ΔVII-19*, *lane 5*) in the absence of tetracycline. Genomic fragments enriched by immunoprecipitation with anti-ETS1 antibody (*lane 3–5*) were PCR amplified with primers specific for EBSiceI (–881 to 696) and EBSiceII (–608 to 408) regions of caspase-1 promoter. The sample from *ΔVII-19* cells was also incubated in the absence of anti-ETS1 antibody and PCR amplified as negative controls (*lane 2*, *No-ab*). Before immunoprecipitation, 5% of the total cross-linked, reversed chromatin from *ΔVII-19* cells was used to amplify EBSiceI and EBSiceII controls (*lane 1*); the same amounts of samples from each cell line were also used to amplify genomic β-actin for the quantification of the genomic DNA present in each sample.