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Characterization of a novel mouse cDNA, ES18, involved in apoptotic cell death of T-cells

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ABSTRACT

Using the modified screening approach in combination with expressed sequence tags, we have identified several novel cDNAs from mouse embryonic stem (ES) cells, whose expression is tissue-restricted and/or developmentally regulated. One of the cDNAs, ES18, is preferentially expressed in lymph node and thymus, and contains noteworthy features of transcriptional regulator. The expression of ES18 transcript was selectively regulated during the apoptosis of T-cell thymoma S49.1 induced by several stimuli. Interestingly, the ES18 transcript was differently regulated in the mutually antagonistic process, between dexamethasone- and A23187-induced cell death of T-cells. Moreover, the message level of ES18 was selectively enhanced by staurosporine, a broad protein kinase inhibitor, but not by other protein kinase inhibitors such as GF109203X and H89. In addition, ES18 transcript was induced by C2-ceramide, which is a mediator of both dexamethasone- and staurosporine-induced apoptotic signaling. We further showed that transient overexpression of ES18 in mouse T-cell lymphoma increased the apoptotic cell death. These data suggest that ES18 may be selectively involved in specific apoptotic processes in mouse T-cells.

INTRODUCTION

Specialization of a cell occurs in response to a series of inductive signals and is accompanied by the expression of specific regulatory genes that determine cell identity. The real key to understanding this specialization may lie in investigating the control of gene expression that results in a variety of developmental phenomena such as changes of cell fate, movement, growth and apoptosis (1). It has been convincingly suggested that the expression of regulatory genes correlates with cell commitment and tissue differentiation in many systems (2–5), and much effort has been directed to identify the genes involved in development, differentiation and apoptosis.

The establishment of embryonic stem (ES) cell lines has opened many approaches in the field of mammalian developmental biology. ES cell lines are totipotent cells derived from the inner cell mass of developing blastocysts (6,7), and retain their totipotential capacity in culture either on embryonic fibroblasts or with leukemia inhibitory factor (LIF) (8,9). Furthermore, ES cells can be induced to differentiate *in vitro*, and form embryoid bodies (EBs) that contain cells of hematopoietic, endothelial, muscle and neuronal lineages (7,10–13). Within EBs, the sequence of events leading to normal differentiation *in vivo* has been observed, suggesting that the *in vitro* model faithfully obeys the rules of the whole animal development. These interesting properties of ES cells provide several advantages for addressing questions related to development, which are difficult, if not impossible, to access *in vivo* (14). One of them is that a large number of cells at important steps along the differentiation pathway could be isolated. This would allow us to enhance our understanding of the rare genetic events in embryonic development. Another important advantage is that the sequential steps of developmental pathway could be investigated in *in vitro* tissue culture. Thus, the ES/EB system is considered a useful model system for the elucidation of the molecular events involved in development, and for the identification of the factors involved in growth and differentiation (15–17).

We surmised that there are a variety of genes that may play important roles in determining the temporal and spatial patterns of embryonic development and tissue differentiation, and that some of them are likely to be expressed in ES cells. Based on such premises, we attempted to identify the new genes with functional roles in differentiation and development from a cDNA library of ES cells. Using the modified screening approach combined with expressed sequence tags (ESTs) (18) and the examination of the expression patterns in adult tissues as well as in *in vitro* differentiation of ES cells, we have isolated and identified several interesting novel cDNA clones meeting these characteristics.

In this report, we describe the characterization of one of the novel cDNAs, designated as ES18, identified by this experimental strategy. ES18 contains several conserved domains found in a number of nuclear regulatory proteins (19). The expression pattern implies that the role of ES18 seems to be associated with

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the immune system, and our results suggest that ES18 may be selectively involved in specific apoptotic pathway and able to effect cell death.

MATERIALS AND METHODS

Accession numbers

The nucleotide sequence for ES18 gene has been deposited in the GenBank database under the accession no. AF083929. The nucleotide sequence of HES18, the human homolog of ES18, can be accessed through the GenBank database under accession no. AF083930.

Reagents

Isoquinolinesulfomide (H89) was purchased from Calbiochem, and [α-^{32}P]dCTP was purchased from Amersham. All other chemicals were obtained from Sigma unless otherwise stated.

Cell culture

The mouse thymoma cell line, S49.1, and lymphoma cell line, EL4, were purchased from ATCC and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The ES cell line D3 was cultured in DMEM supplemented with 15% heat inactivated fetal bovine serum, LIF (1000 U/ml, Gibco BRL), and 150 mM monothioglycerol in tissue culture dishes that were pre-coated with 0.1% 300-bloom porcine gelatine (Sigma).

In vitro differentiation of ES cells

To induce *in vitro* differentiation of ES cells, rapidly growing colonies of undifferentiated ES cells were trypsinized with 0.25% trypsin and 1 mM EDTA and transferred to bacterial culture dishes in a medium without LIF. To standardize cell input for the induction procedure, one-quarter of the cells from a single confluent 90 mm tissue culture dish were suspended in 10 ml of medium. The media were replaced with fresh media after 2 days.

Screening of cDNA library and computer analysis

Using poly(A)$^+$ RNA from mouse ES cells, a cDNA library was constructed in the λ ZAP vector following the manufacturer's protocol (Stratagene). The library was converted to pBluescript plasmids, transfected into *Escherichia coli* XLI-Blue (Stratagene) cells. After choosing clones from the library in an unbiased manner, the subtraction of rRNA contaminants were performed by plaque hybridization with a slight modified grid transfer using ^{32}P-labeled 18S RNA, 28S RNA and 5S RNA as probes. Clones were selected and their nucleotide sequences were determined by single-run sequencing (18). The EST sequences were examined for similarities using the BLAST network service at NCBI. Slot blot analysis was performed on the new genes of the sequence generated. The ^{32}P-labeled cDNA probes were prepared from poly(A)$^+$ RNA of the various adult tissues, ES cells and *in vitro* differentiated ES cells (day 2, 4, 6, 8, 10, 12).

Cloning of the ES18 cDNA

To isolate the full gene of ES18, the mouse thymic cDNA library (Stratagene) was re-screened with the 1.2 kb cDNA fragment of ES18 as a probe, and several overlapping cDNA clones containing the full gene or partial fragment of ES18 were isolated. The partial clone of human ES18 homolog was also isolated by screening of the human thymic cDNA library (Stratagene) using the same probe.

Northern blot analysis

Total RNA was extracted with guanidium isothiocyanate. The RNA was separated on 1% agarose gel containing formaldehyde, transferred to nitrocellulose membranes (S&S) and hybridized with ^{32}P-labeled 330 bp fragment (18*Xba*I, bases 1199–1530) of ES18. Hybridization was performed at 42°C in a buffer containing 50% formamide, 5× Denhardt's solution, 5× SSC, 0.1% SDS, 100 µg/ml of salmon sperm DNA, 100 µg/ml of yeast tRNA, and ^{32}P-labeled probe. The filter was then washed with 2× SSC, 0.1% SDS and 0.1× SSC, 0.1% SDS.

DNA fragmentation assay

The cells were harvested after appropriate drug treatment, washed once with ice-cold PBS, and resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.5% sodium lauryl sarcosine) containing 100 µg of proteinase K. The mixture was incubated at 55°C for 2 h. The DNA was extracted with phenol/chloroform and precipitated with ethanol. After washing once with 70% ethanol, the samples were digested with 0.1 µg of RNaseA at 37°C before being loading onto 1.2% agarose gels, and the DNA was visualized by ethidium bromide staining.

Electron microscopy

S49.1 cells treated with staurosporine (100 nM) were fixed in 4% paraformaldehyde and 5% glutaraldehyde in cacodylate buffer (pH 7.4). Cells were post-fixed in 2% buffered osmium tetroxide. After staining en bloc in 0.4% uranyl acetate, cultures were dehydrated serially through increasing concentrations of ethanol and embedded in Epon resin (EMbed-812, Electron Microscopy Sciences). Ultrathin sections (70 nm) were prepared on Reichart-Jung Ultracut J, picked up on collodion-coated copper grids, and double-stained with 0.4% uranyl acetate and 2% lead citrate. After carbon coating, the samples were observed on JEOL 1200EX-II electron microscope.

Assay of apoptosis

Apoptosis was induced by incubating cells with various reagents and overall cell injury was estimated by examination of cultures with phase-contrast microscopy. The apoptotic cell death was also measured by flow cytometry as described by Nicoletti *et al.* (20). Briefly, the harvested cells were fixed with 70% ethanol and stained with 50 µg/ml of propidium iodide (PI). The cells were treated with 10 µg/ml of RNaseA for 30 min at 4°C. After washing with PBS buffer, stained cells were analyzed using the FACStar^{plus} (Becton Dickinson) for the DNA content.

Transient transfection assay

To construct the eukaryotic expression vector for ES18, a 2.4 kb *Xho*I fragment containing the full-length coding sequence and 3′ untranslated region of the ES18 was inserted into the pcDNA 3.1(+) (Invitrogen) in the sense and antisense orientation. The resulting plasmids were designated as pcDNA ES18S and pcDNA ES18AS, respectively. Transient transfections into EL4, a lymphoma cell line,

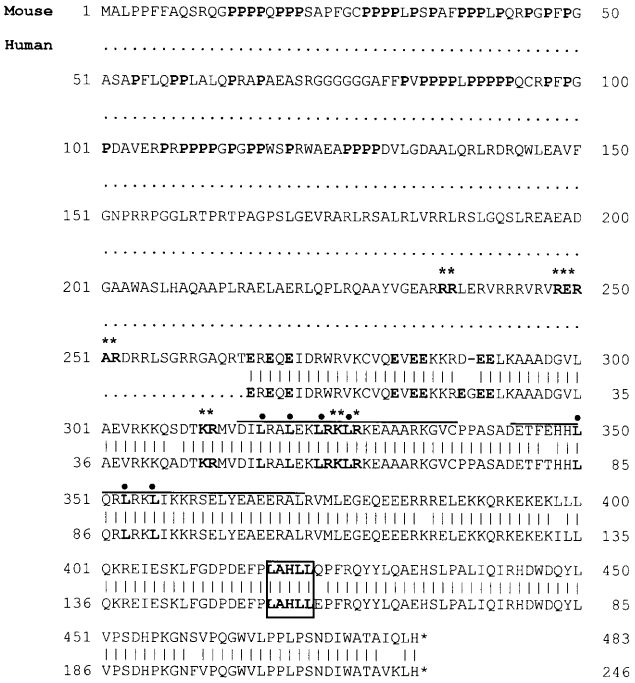

Figure 1. Amino acid sequences of murine ES18, in comparison with the human counterpart. The PSORT program predicted the nuclear targeting sequences [2 basic-10 spacer-5(3)basic], shown with asterisks. The putative LXXLL nuclear receptor binding motif is boxed, and proline and glutamic acid residues belonging to the proline-rich regions and acidic-rich domain are highlighted by bold face. The α-helix regions with evenly spaced leucine residues (•) are underlined. Sequence alignment was generated with the DNAStar program, which demonstrates a high degree of conservation of the ES18 sequence between mouse and human.

were performed as described by Grosschedi and Baltimore (21) using a DEAE-dextran/chloroquine procedure. Cells are passed over Percoll to remove dead cells 24 h after transfection (22). EL4 cells were cotransfected with pEGFP expression vector and test plasmids, and successfully transfected cells were analyzed using green fluorescent protein (GFP) fluorescence.

RESULTS

Screening for the genes with tissue-restricted and developmentally regulated expression

To isolate the novel genes involved in early development and tissue differentiation, we used the modified screening approach in combination with the EST. EST was initially performed on the randomly selected clones from cDNA library of mouse ES cells, as outlined in Materials and Methods. After analysis by BLAST homology search, 41 clones that were either novel or matched only to EST sequences were investigated using slot blot analysis. To see whether they represented differentially expressed genes, the probes were designed to examine the expression patterns in various adult tissues and during the *in vitro* differentiation of ES cells. Using ^{32}P-labeled cDNA probe generated from poly(A)$^+$ RNA of the adult tissues and *in vitro* differentiated ES cells (day 2, 4, 6, 8, 10 and 12), we were able to isolate several cDNA clones of interest. One of them, #18 clone, hereafter referred to as ES18, was further characterized because of its highly specific expression in lymph node and thymus.

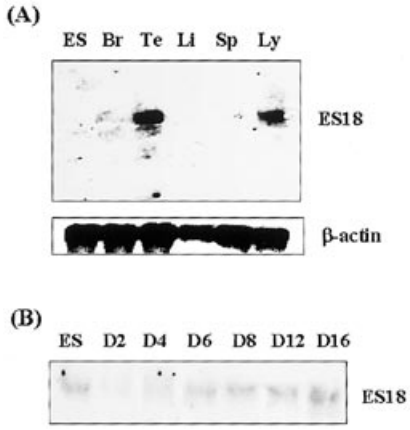

Figure 2. Expression of ES18 in adult tissues and during *in vitro* differentiation of ES cells. (**A**) Each lane contains 15 µg of total RNA from various tissues: ES, embryonic stem cells; Br, brain; Te, testis; Li, liver; Sp, spleen; Ly, lymph node. The blot was hybridized with ^{32}P-labeled 330 bp fragment (bases 1199–1530) of ES18 (ES18*Xba*I) as a probe. The same blot was re-probed with the β-actin cDNA fragment as a control. (**B**) Thirty micrograms of total RNA were isolated from EBs at advancing days (D) of *in vitro* differentiation of ES cells and then hybridized with ES18*Xba*I probe.

Cloning and characterization of cDNA clones for ES18

Sequence analysis of ES18 cDNA, containing a 1.2 kb insert, revealed that the 5′ end of this gene was missing. To fully characterize ES18, the thymic library was re-screened with a 5′-330 bp fragment (ES18*Xba*I) of #18 clone as a probe. Successive screenings of this library led to the identification of a series of highly overlapping clones that contained additional fragments. A represented full-length clone has a 2.4 kb insert that contained an open reading frame (ORF) of 1449 bp encoding 483 amino acids. Comparison of the sequence with those available in the database did not reveal any genes with extensive homologies. However, the deduced amino acid sequences showed the noteworthy features of transcriptional regulators (19) (Fig. 1). The proline-rich regions with several short stretches of proline residues are found in the N-terminal part, which was followed by acidic regions with high contents of glutamic acid (E). The Chou–Fassman and Garnier–Osguthrope–Robson algorithms predict several α-helix regions in the C-terminal part, and several evenly spaced leucines and hydrophobic amino acids with the potential for coiled-coil interactions are located in a putative paircoil region. The LXXLL motif, which is a signature motif for transcriptional co-activators and known to mediate binding to the nuclear receptors (23), is also found in the C-terminal half. Searches with PSORT program shows nuclear localization signals [2 basic-10 spacer-5(3)basic]. In addition, the partial clone of human ES18 homolog containing 219 amino acids was also isolated from the human thymic cDNA library using the same probe. The murine and human ES18 are highly conserved, being 95% homologous with only 10 amino acid changes (Fig. 1).

Northern blot analysis was performed with the RNAs from various adult tissues to confirm our original observation. The result demonstrated that ES18 was mainly expressed in testis, lymph node (Fig. 2A) and thymus. To determine whether the ES18 is differentially expressed during the *in vitro* differentiation of ES cells, blot containing RNA from ES cells and EB was sequentially hybridized to ES18*Xba*I fragment (bases 1199–1530). As shown in

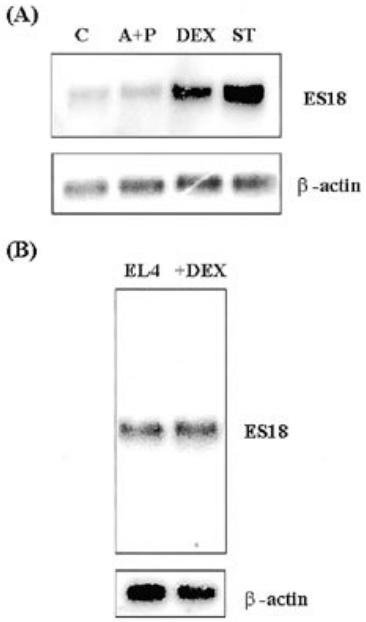

Figure 3. Expression of the ES18 mRNA in apoptotic T-cell thymoma. (**A**) Immature T-cell thymoma S49.1 cells were treated with A23187 plus PMA (A+P), dexamethasone (DEX) and staurosporine (ST) for 12 h. (**B**) Mature T-cell lymphoma EL4 cells were treated with dexamethasone for 12 h. Thirty micrograms each of total RNA were electrophoresed, transferred to nitrocellulose membrane, and hybridized with ES18*Xba*I probe.

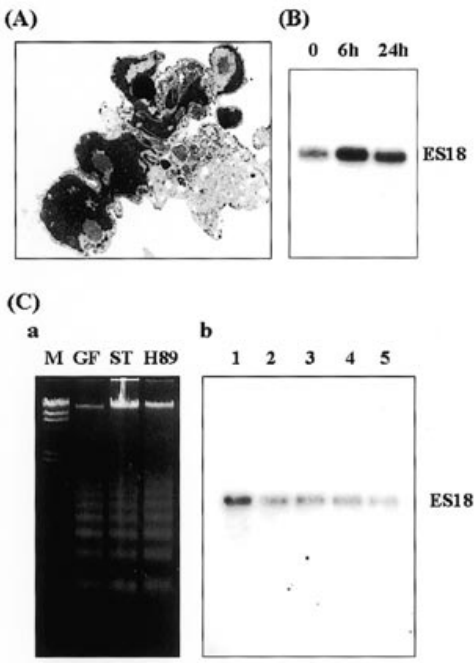

Figure 4. Selective induction of ES18 mRNA in staurosporine-induced apoptosis. (**A**) Electron microscopic analysis of apoptotic S49.1 cells by staurosporine (100 nM). Apoptotic morphology was investigated 24 h after addition of staurosporine to culture at concentration of 100 nM. (**B**) Time course analysis of the level of ES18 transcript in staurosporine-induced apoptotic cells. (**C**) Expression of the ES18 transcript in apoptotic cells treated with several protein kinase inhibitors. S49.1 cells were treated with GF109203X (5 μM), H89 (10 μM), PMA (12 nM) for 12 h. a, effect of protein kinase inhibitors on DNA fragmentation. M, λ *Hin*dIII marker. b, northern blot analysis of ES18 mRNA level: 1, staurosporine; 2, control; 3, GF109203X; 4, PMA; 5, H89.

Figure 2B, a very low level of ES18 was detected. These data suggest the possibility that the expression of ES18 transcript may be associated with the immune system. On this ground, we have made attempts to determine the identity of ES18 in the immune system.

Expression of the ES18 in apoptotic T-cell thymoma

Apoptosis is one of the major phenomena observed in developing thymocytes and also in peripheral T-cells to maintain homeostasis (24). In an attempt to identify the function of ES18 in the immune system, we investigated the transcriptional regulation of ES18 during the apoptosis of T-cell thymoma S49.1 induced by several stimuli. The level of ES18 mRNA was significantly increased in dexamethasone (1 μM)- or staurosporine (100 nM)-induced apoptotic cells. However, ES18 transcript was not induced in phorbol-12-myristate 13-acetate (PMA, 12 nM) plus A23187 (1 mM)-treated cells, which mimics signaling via TCR/CD3 complex (Fig. 3A). The same result was also observed in T-cell thymoma 16A5 (data not shown). Moreover, dexamethasone-treated T-cell lymphoma EL4, which is resistant to the apoptosis-inducing activity of glucocorticoid, did not induce the expression of ES18 gene (Fig. 3B). Thus, the expression of ES18 seems to be related to glucocorticoid-induced apoptosis. It is noteworthy that activation driven cell death and glucocorticoid-induced apoptosis pathways are mutually antagonistic (25). Several genes have been implicated in these antagonistic apoptotic process. For example, Nurr77 is uniquely induced in the TCR/CD3 apoptotic cell death of thymocytes, but not in glucocorticoid-mediated cell death (26,27). These results led us to look at the role of the ES18 in specific apoptotic process.

Regulation of ES18 mRNA expression in staurosporine, a protein kinase (C) inhibitor, induced apoptosis

Staurosporine, a broad protein kinase inhibitor, has been known as a potent inducer of apoptosis in many different cell types (28,29). We confirmed that staurosporine also induced apoptosis in S49.1 cells by electron microscopy (Fig. 4A), and further examined the regulation of ES18 transcript in this staurosporine-induced apoptotic process. The induction of ES18 transcript was clearly visible in staurosporine-treated cells within 6 h (Fig. 4B). Many reports have suggested that the inhibition of protein kinase is closely associated with apoptotic signaling pathway (30,31). Therefore, we surmised that the up-regulation of ES18 may be mediated through the inhibition of protein kinase. To test this possibility, the effects of several protein kinase inhibitors, known to exert effects on apoptosis, were analyzed. The signs of apoptosis, detectable by morphological changes and DNA ladder formation, became apparent in 12 h upon treatment of all protein kinase inhibitors used in this experiment (Fig. 4C, a), whereas GF109203X, a protein kinase C inhibitor, and H89, a protein kinase A inhibitor, failed to induce the ES18 transcript (Fig. 4C, b). These data indicate that ES18 may be specifically involved in the staurosporine-induced apoptotic pathway, independently of protein kinase inhibition.

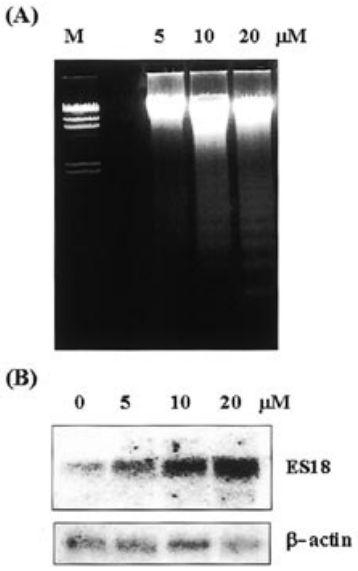

Figure 5. Effects of cell-permeable ceramide analog (C2) on the expression of ES18 transcript in immature T-cell S49.1. (**A**) Exogenous C2-ceramide dose-dependently stimulated cell death. S49.1 cells were incubated for 12 h in the presence of indicated concentrations of C2-ceramide. Cell death was quantitated by the DNA fragmentation assay. M, λ *Hin*dIII marker. (**B**) Dose response for induction of ES18 transcript by C2-ceramide. S49.1 cells were treated with C2-ceramide at various concentrations for 12 h, and then the transcript level of ES18 was analyzed by northern blot hybridization. Total RNA was extracted, and 30 μg per lane was used for the analysis.

Effects of ceramide on the expression of ES18 transcript in S49.1 immature T-cell

There are many reports that the apoptotic pathway by staurosporine appears unique among other protein kinase inhibitors (28,32,33), and the formation of ceramide is one of the essential events in the staurosporine-induced cell death. Ceramide has been strongly implicated in the process of apoptosis induced by TNF-α, Fas ligands, ionizing radiation, dexamethasone, and so forth (34). For this reason, we investigated the effect of exogenous ceramide on the expression of ES18 in immature T-cell S49.1 and mature T-cell EL4 (data not shown). To correlate ES18 gene expression with ceramide-induced apoptosis, we examined the level of ES18 mRNA after treatment with various concentrations of ceramide. In S49.1 cells, the effects of C2-ceramide were first detected after 2 h (data not shown), and increases in C2-ceramide concentrations resulted in increased DNA fragmentation (Fig. 5A). Coincidentally, the level of ES18 transcript was dose-dependently induced by C2-ceramide (Fig. 5B). These results suggest the possibility that the specific induction of ES18 mRNA in apoptotic cells may be linked to ceramide-mediated signal.

ES18 overexpression induces apoptosis

We then focused our attention on the question of whether ES18 is able to effect or prevent cell death. To gain insight into the effect of ES18 expression on the apoptosis of T-cells, plasmids expressing the ES18 gene containing the full-length ORF in sense (pcDNAES18S) and antisense (pcDNAES18AS) orientations were constructed, and tested in a transient transfection assay for their ability to relate with the apoptotic process. Transfected cells were compensated by co-transfection with a plasmid containing the GFP (pEGFP). In addition, we confirmed whether pcDNAES18S was expressed in transiently transfected cells by northern blot analysis (data not shown). Successfully transfected cells were analyzed by flow cytometry and fluorescent microscopy on the basis of GFP fluorescence. As presented in Figure 6A, the FACS analysis showed that the fraction of apoptotic cells was ~3–5% in the transfected cells with vector alone (pcDNA 3.1) or ES18 antisense (pcDNAES18AS), whereas it was increased up to 19% in cells expressing ES18 in the sense (pcDNAES18S) orientation. In addition, the treatment of actinomycin-D could inhibit apoptotic cell death induced by ES18, indicating that ES18 mediates apoptosis through a transcription-dependent mechanism (data not shown). These results were further supported by the microscopic analysis. Many of the fluorescent cells transfected with pcDNAES18S exhibited morphological characteristics of apoptotic cell death (Fig. 6B). By contrast, transfection with vector alone (pcDNA 3.1) revealed mainly healthy looking green fluorescent cells (Fig. 6B). These observations further suggest that the expression of ES18 is associated with the apoptotic cell death.

DISCUSSION

Our experimental approach was designed to identify the novel genes involved in early development and tissue differentiation in mouse. For exploring the molecular events in early development of mouse, the capacity of totipotent ES cells was employed. ES cells retain their totipotency in culture and differentiate *in vitro* into cells of multiple embryonic lineages. On the basis of the expression patterns in adult tissues and during *in vitro* differentiation of ES cells, we have isolated and identified several interesting novel cDNA clones from cDNA library of ES cells. In this paper we report the identification of one of the novel cDNAs, named as ES18, which is predominantly expressed in lymph node, thymus and testis.

The ES18 cDNA contains a 1449 bp ORF capable of encoding a 55 kDa protein, and an antibody, raised against the predicted ORF, recognizes a protein of similar size (data not shown). Analysis of the predicted protein shows potential structural features present in many transcriptional regulators (19), including proline-rich domains, acidic regions with high contents of E, putative nuclear localization signals, several α-helix regions and LXXLL motif. The LXXLL motif is a signature motif in nuclear proteins such as transcriptional coactivators, and a short sequence motif is necessary and sufficient to mediate binding to liganded nuclear receptors (23). This motif has been found in several transcriptional regulators such as RIP140 (35), CBP (36)/P300 (37), Trip (38) and TIF (39). In addition, it is noteworthy that the potential pair coil region with evenly spaced leucine residues shows homology to the helix region of yeast A42 mating type factor (34% identity, 54% positive similarity) (40) and rat thyroid-specific transcription factor 1-homeodomain (30% identity, 75% positive similarity) (41). Although there is no definite evidence, the above structural feature of predicted ES18 gene product implies the possibility that it plays a regulatory role.

It is notable that the ES18 transcript is preferentially expressed in thymus and lymph node, and that a very low level of ES18 is detected during the *in vitro* differentiation of ES cells (Fig. 2). It is tempting to speculate that the function of ES18 may be linked to the immune system. Among the several possibilities for the role of ES18 in the immune system, we first investigated the possibility that ES18 is associated with apoptosis. Apoptosis is a

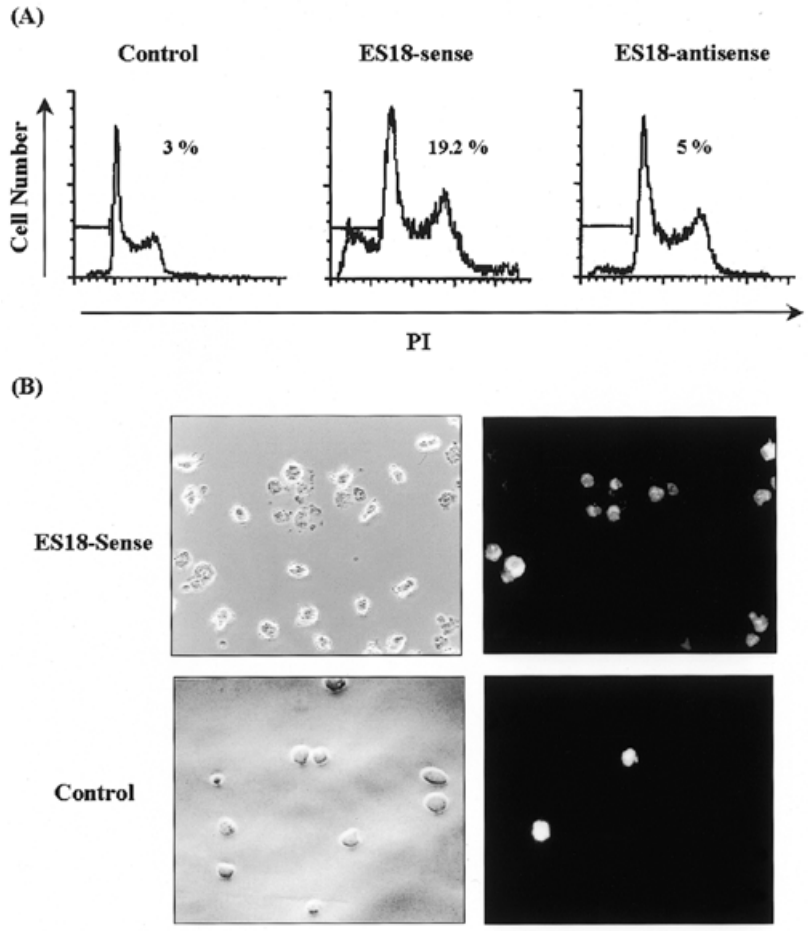

Figure 6. ES18 overexpression induces apoptosis. Mature T-cell, EL4 cells were co-transfected with the pEGFP expression vector and pcDNA3.1 control vector, ES18-sense, and ES18-antisense construct. (**A**) Cell cycle profile of the transiently transfected cells. After 48 h of transfection, the cells were stained with 50 µg/ml PI, and analyzed on the basis of GFP expression using the FACStar^{plus} (Becton Dickinson). The bar represents apoptotic cell populations. (**B**) Cells were simultaneously evaluated by phase contrast microscopy (left) and by fluorescence microscopy (right) to detect apoptotic cells.

physiologically regulated cell death involved in the normal development and the homeostasis of adult state. During development and homeostasis of the immune system, B-cell and T-cell lineages undergo selection that involves massive cell death (24). For this reason, the transcriptional regulation of ES18 transcript in the apoptotic T-cell thymoma S49.1 by several stimuli was examined. The results supported our assumption. Northern blot analysis showed that the ES18 transcript was differentially regulated in several apoptotic stimuli (Fig. 3). The result seen in dexamethasone- and A23187 plus PMA-induced apoptotic cells seems more interesting in a point of view that TCR/CD3-driven cell death and glucocorticoid-induced death are mutually antagonistic. It has been convincingly reported that TCR/CD3-driven cell death and glucocorticoid-induced death utilize different sets of mediators, with each stimulus blocking the activity of the mediator induced by the other (25,42). When the apoptosis was induced in S49.1 cells by dexamethasone, significant elevation of ES18 transcript was observed at 6 h (data not shown). However, the transcript was weakly down-regulated by A23187 plus PMA. It seems conceivable that ES18 may be selectively involved in specific apoptotic pathway, and this idea is further supported by the following results.

ES18 transcript was up-regulated by staurosporine, a broad protein kinase inhibitor. This result raised the possibility that the regulation of ES18 could be mediated by the inhibition of protein kinase. After confirmation that staurosporine induced apoptosis in S49.1 cells by electron microscopy (Fig. 4A), it was further tested using other specific protein kinase inhibitors. However, GF109203X, a protein kinase C inhibitor, and H89, a protein kinase A inhibitor, both failed to enhance the ES18 transcript (Fig. 4B). A possible explanation for this result could be that up-regulation of ES18 by staurosporine may not be related to inhibition of protein kinase. There are many reports that the staurosporine induces the apoptosis via distinct pathways independent of protein kinase inhibition, and formation of ceramide is one of the essential events in the staurosporine-induced cell death (26,32).

Ceramide is known as a mediator involved in the regulation of important cell functions including cell growth, differentiation and apoptosis (43). A number of stimuli such as TNF-α, Fas ligands, ionizing radiation, serum withdrawal, dexamethasone and staurosporine were shown to cause an elevation in the endogenous levels of ceramide (34). We therefore considered the possibility that the expression of ES18 in apoptotic cells was related to the ceramide-mediated signaling. To address this question, we

examined the effect of ceramide on the expression of ES18 in apoptotic S49.1 cells. The elevation of ES18 transcript was detectable 2 h after treatment of C2-ceramide (data not shown), and the level of the transcript was dose-dependently increased (Fig. 5). The correlation of apoptosis with ES18 expression was measured by DNA fragmentation assay. These findings imply that the specific induction of ES18 transcript seems to be related to ceramide-mediated signal.

Further support for ES18 being involved in apoptosis is provided by the transient transfection analysis. We investigated whether ES18 could effect or prevent apoptotic cell death by overexpression of ES18 gene in T-cell lymphoma EL4 (Fig. 6). In ES18 overexpressed cells, the apoptotic population was significantly increased, compared to the cells with vector alone or antisense orientation. In fluorescent microscopy and flow cytometry analysis, many of the transfected cells with ES18 in sense orientation showed the apoptotic characteristics. This result implies that ES18 seems to be one of the genes involved in stimulating the apoptotic cell death. It has been reported that apoptosis must require new gene expression in order to proceed. Indeed, a number of genes with properties of transcription factors have been identified that can trigger apoptotic cell death (44,45). They are also known to be involved in other cellular processes such as cell cycle progression, transformation and differentiation, or they may be specific to cell death (46–48). Recently, we observed that stable cell lines overexpressing ES18 in NIH 3T3 fibroblasts displayed anchorage-independent growth in soft agar assays (in preparation). This finding raises the interesting possibility of speculating about the function of ES18. Many reports have suggested that the cellular pathways controlling apoptosis are interlinked with those controlling proliferation and differentiation. Our study at present is not sufficient to pinpoint the role(s) of ES18 in the immune system, and further experiments are currently in progress to define it. Taken together, our characterization of ES18 strongly suggests that it may be selectively involved in specific apoptotic process in T-cell lines.

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