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Down-regulation of *Tcf*-1 Expression by Activation-induced Apoptosis of T Cell Hybridoma

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The *Tcf*-1 (T cell specific factor-1) is a transcription factor uniquely expressed in T-lineage cells. Its expression is developmentally regulated, which is high in the specific stage of immature thymocytes, but is much lower in mature T cells. We cloned the *Tcf*-1 gene by subtractive hybridization and found it to be highly expressed in the thymus compared to the mRNA level in the spleen as expected. Since apoptosis occurs enormously in the thymus, we were interested in whether *Tcf*-1 gene expression could be regulated by such a high level of apoptotic assault. By using T cell hybridoma 70.7 cells, we induced apoptosis by incubating cells with anti-CD3 antibody *in vitro*. After apoptosis induction, *Tcf*-1 mRNA level was found to be significantly reduced compared to normal cells. Since *Tcf*-1 is a transcription factor for the CD3-ε gene, we tested how CD3-ε expression is regulated in apoptotic cells. The surface level of CD3-ε protein is also down-regulated after apoptosis induction. Such a down-modulation of CD3-ε protein would reduce the TCR/CD3 complex on the cell surface, which would be an important regulator for T cell apoptosis.

Apoptosis plays important roles in the immune system (Cohen and Duke, 1992; Schwartz and Osborne, 1993). Its role includes not only shaping of immune repertoire by positive/negative selection in the thymus, but also regulating immune activity in the periphery. In particular, because more than 95% of thymocytes die in the thymus, it is known that apoptosis is an essential process in positive/negative selections (Iwata et al., 1991). The positive selection of immature thymocytes seems to be related to the protection offered by Bcl-2 during apoptotic assault generated by a high concentration of the glucocorticoid hormone (Nunez and Clarke, 1994; Reed, 1994; Memon et al., 1995). The negative selection in the thymus seems to result from the activation by ligating T cell receptor (TCR), which eventually leads to apoptosis of thymocytes. In addition to the apoptosis of immature thymocytes in the thymus, mature T cells also go through cell death in the periphery as a way of regulating immune responses. It also involves activation-induced apoptosis to remove the clones expressing the specific TCR/CD3 complex. Due to the complexity of apoptotic processes *in vivo*, *in vitro* system was developed to mimic them; apoptosis of thymocytes can be induced either by glucocorticoids or by antibodies to the TCR/CD3 complex (Jondal et al., 1995; Montague and Cidlowski, 1995). An increase of intracellular Ca^{2+} concentration with various agents, including Ca^{2+} ionophore, has also been shown to cause apoptosis in thymocytes. Such a Ca^{2+}-dependent apoptosis seems to be either via the glucocorticoid pathway or via the TCR-mediated pathway (McConkey et al., 1989; Cohen and Duke, 1984). By using these procedures, molecular mechanisms underlying cell death of self-reactive T cells have also been studied, but not fully understood (Park et al., 1995; Woronicz and Winoto, 1995). T cell hybridomas have been used as a useful model system to study apoptosis *in vitro* because the glucocorticoids-, or TCR-mediated apoptosis can be induced in these cells. Activation-induced apoptosis in T cell hybridomas has been especially useful as a model for negative selection in the thymus and for extrathymic deletion in the periphery. From the studies utilizing this system, it now seems clear that the activation-induced apoptosis in the periphery is mediated by the up-regulation of Fas and Fas ligand (Osborne and Schwartz, 1994; Brunner et al., 1995; Cleveland and Ihle, 1995) and by the activation of caspase proteases (Nicholson and Thornberry, 1997). Great advances have been made recently to identify the genes involved and to characterize their roles in apoptosis (Nagata, 1997; Peter et al., 1997), but more studies are needed to understand the exact mechanism of apoptosis of T cells.

In this study, we attempted to identify genes involved in apoptosis of T cells. Since it is known that

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apoptosis occurs at a greater extent in the thymus, we started by isolating genes highly expressed in the thymus. One of genes identified by this strategy encodes the transcription factor, previously known as a *Tcf*-1 (T cell factor-1) (van de Wetering et al., 1991). *Tcf*-1 was originally cloned because of its affinity to the AACAAAG motif in the enhancer of the *CD3-ε* chain gene. It was later found that it also could bind to the moderately degenerate heptamer motif A/T A/TCAAAG in other T cell specific genes such as TCR-α, -β, -δ and CD4 (Oosterwegel et al., 1991a; Oosterwegel et al., 1991b; Clevers et al., 1993). Since its target genes, TCR, CD4 and CD3, are known to be important for T cell differentiation and activation, it would be reasonable to expect that the *Tcf*-1 transcription factor might have a great impact on the regulation of T cell activities (Leiden, 1992). Another transcription factor, *Lef*-1 (Lymphoid enhancer-binding factor), is highly related to *Tcf*-1 and it was shown to have a similar binding specificity in lymphoid specific genes (Clevers et al., 1993). Sequence analysis of *Tcf*-1 and *Lef*-1 indicated the presence of a single HMG-1 (High Mobility Group) box sequence, which is known to mediate the sequence specific DNA binding in various proteins. Even though the exact mechanism of *Tcf*-1 mediated transcriptional activation is not currently known, some evidence implies that *Lef*-1 binds to the minor groove of double helix and bends DNA in the enhancer of the TCR-α gene (Love et al., 1995). Therefore, the *Tcf*-1 protein might act as an architectural coordinator of the multiprotein enhancer-binding complex to recruit other components of the complex.

Expression of *Tcf*-1 is unique in that it is only detected in the T lineage cells, whereas its close relative, *Lef*-1, is expressed in B cells as well as in T cells (Oosterwegel et al., 1993). Developmental expression of *Tcf*-1 was studied throughout thymocyte differentiation. Its expression is very low at an early stage of thymocyte development in double negative (DN) thymocytes when CD3 and TCR gene expressions are also low. However, in immature single-positive (ISP) thymocyte, *Tcf*-1 expression is at the highest level and declines thereafter. Later in development, mature T cells in the periphery seem to have a much lower level of *Tcf*-1 mRNA (Verbeek et al., 1995). Thymocytes from *Tcf*-1 and/or *Tcf*-1/*Lef*-1 double knockout mice showed very scarce single positive (SP) and double positive (DP) thymocyte population; the majority of thymocytes from these mice are at DN stages (Verbeek et al., 1995; Okamura et al., 1998). These results suggest that *Tcf*-1 might play a crucial role in T cell development.

In this study, we tested the expression of the *Tcf*-1 gene during apoptosis of T cell hybridoma, 70.7 cells. In addition, because *Tcf*-1 regulates genes which are important for T cell activation, we tested whether down-regulation of *Tcf*-1 expression also affects the expression of its target genes, CD3-ε, during this process. Possible mechanisms of apoptosis involving the *Tcf*-1 transcription factor will be discussed.

Materials and Methods

Cell lines, reagents and antibodies

The murine T cell hybridoma 70.7 is a D10 TCR transfectant of 4G4 and was originally obtained from C. Janeway at Yale University. The 70.7 cells were maintained in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS). The calcium ionophore (A23187) and propidium iodide (PI) were purchased from Sigma Chemical Co. The anti-CD3 antibody (145-2C11) were purified and conjugated with FITC.

Isolation of Tcf-1 clone

Thymus specific genes were cloned by the subtractive hybridization strategy with spleen mRNA and thymus cDNA. Intact thymi and spleens were collected from 3 to 5 wk old mice and used as sources for RNA. Total RNA was isolated by $CsCl_2$ banding and poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (Aviv and Leader, 1972; Chomczynski and Sacchi, 1987). About 10 mg of poly(A)$^+$ RNA were heated at 65℃ for 2 min and annealed with 1 mg of magnetic Dynabeads oligo$(dT)_{25}$ (Dynal) for 30 min at room temperature. The annealed poly(A)$^+$ RNA was separated by a magnetic field used as templates for the synthesis of the first-stranded cDNA from the thymus and spleen. The second stranded cDNA was synthesized by a random priming method with hexanucleotides and 200 μCi [α-^{32}P]dCTP (3000 Ci/mmol). In order to prepare a subtractive probe, the first stranded spleen cDNA which were conjugated with magnetic beads were mixed with the labeled thymic probe, and incubated at 55℃ for an hour. The labeled thymic probe which was hybridized to the first stranded spleen cDNA was removed using a magnetic field, and the remaining single-strand labeled DNA was used as a subtractive probe to screen thymic cDNA library (from M. M. Davis, Stanford University). Many clones were identified in the first screening, but additional screening by differential hybridization with spleen and thymus probes identified three independent clones, one of which being a *Tcf*-1 gene.

DNA sequencing and computer analysis

To determine the nucleotide sequences, the restriction fragments of the cloned gene were subcloned into the pBluscript SK(-) vector (Stratagene). The nucleotide sequence was determined by dideoxy chain termination method using a Sequenase 2.0 kit (United States Biochemical). Comparisons of the nucleotide sequence with sequences in GenBank were performed at the National Center for Biotechnology Information using the BLAST network service.

Induction and measurement of apoptosis

Apoptosis of T cell hybridomas was induced by incubating cells with anti-CD3 antibody. Briefly, the anti-CD3 antibody, 145-2C11, was coated to 6-well plates by incubating at 37℃ for 90 minutes. After overnight incubation at 4℃, the unbound antibody was washed with PBS. Then cells were incubated in the anti-CD3 coated plate at 10^5 cells/ml for 3 d and harvested to measure the extent of apoptosis. A23187 was also used to induce apoptosis. For this, the 70.7 cells (10^5 cells/ml) were treated with A23187 dissolved in ethanol at a final concentration of 1 μM. After this apoptotic assault, cells were incubated for various times, usually up to 24-48 h. To detect apoptotic cell death, a DNA fragmentation assay was employed. Briefly, at the end of incubation, cells were lysed in lysis buffer (10 mM EDTA; 50 mM Tris-HCl, pH 8.0; 0.5% lauryl sarcosine; 100 mg/ml proteinase K) at 55 ℃ for 2 h and then extracted with phenol/chloroform. After RNase A treatment, DNA was analyzed by 2% agarose gel electrophoresis. The second method involved the utilization of flow cytometry. After harvesting, cells were fixed with 70% ethanol, stained with 50 μg/ml of propidium iodide and treated with 10 μg/ml of RNase A. The extent of apoptosis was estimated by measuring the degree of DNA content as described by Nicoletti et al. (1991).

Flow cytometry

The FACStar flow cytometer (Becton and Dickinson) was used to analyze the fluorescence of cells labeled with propidium iodide (PI) and FITC-labeled anti-CD3 antibody. For measuring the surface level of the CD3-ε protein in apoptotic cells, the 70.7 cells were treated with A23187 for 24 h to induce apoptosis, and stained with 145-2C11 against the CD3-ε protein. These cells were fixed with 70% ethanol, and then stained with propidium iodide to label DNA. Cells with diploid or subdiploid DNA content were analyzed for the surface expression of CD3 protein by setting gates for the cells with different PI intensity on FACS analysis.

Results

Reduction of Tcf-1 expression following anti-CD3 antibody treatment

To find genes which might be important for the T cell differentiation in the thymus, subtractive hybridization was performed to obtain thymus-specific genes as described in Materials and Methods. Differential hybridization with spleen and thymus probes revealed a couple of clones containing the *Tcf*-1 (T cell factor-1) cDNA (Fig. 1A). Northern blot analysis with *Tcf*-1 cDNA probe confirmed the high expression of *Tcf*-1 mRNA in the thymus, but low expression in the spleen (Fig. 1B). This result is in good agreement with

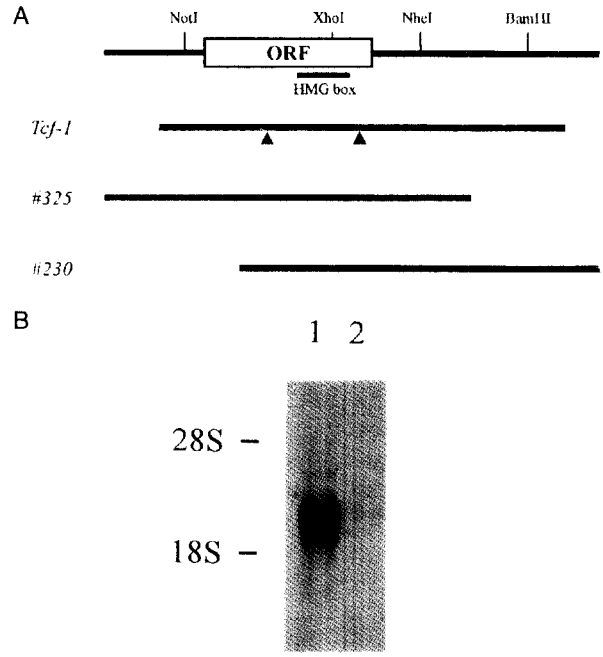

Fig. 1. A, cDNA clones containing the coding region of the *Tcf*-1 gene. The #325 and #230 clones were ligated in the NheI site and the full length of the *Tcf*-1 gene containing a 2.6 kb of insert which contains more sequences of *Tcf*-1 gene than originally reported, was constructed. The closed triangles indicate the alternative splicing site of *Tcf*-1 gene. B, Expression of *Tcf*-1 mRNA in the thymus (lane 1) and spleen (lane 2). The same amount (10 μg) of total RNAs were loaded in each lane of gel electrophoresis, and hybridized with the ^{32}P-labeled 1.3 kb XhoI fragment of *Tcf*-1 gene as a probe.

developmental expression of *Tcf*-1 mRNA; its expression is known to be maximal in immature thymocytes, but at a reduced level in the peripheral mature T cells (Oosterwegel et al., 1993; Verbeek et al., 1995). Since *Tcf*-1 mRNA is uniquely expressed during thymocyte development as described above (Verbeek et al., 1995), one of the functions of the *Tcf*-1 protein could be involved with apoptosis, which occurs at an enormously high level in the thymus. To see how *Tcf*-1 expression is altered by apoptosis, T cell hybridoma, 70.7 was used as a model system, in which apoptosis can be induced by various ways. We treated T cell hybridoma with anti-CD3 antibody to induce apoptosis (Smith et al., 1989); it activates T cells by TCR/CD3 complex engagement followed by apoptosis of the cell. Such a treatment leads to an activation-induced apoptosis, which is known to operate in the negative selection of the thymus and in the deletion of activated T cells in the periphery.

After stimulation of T cells with anti-CD3 antibody, genomic DNA was extracted from the cells and examined for DNA fragmentation, a hallmark of apoptosis (Wyllie, 1980; Wyllie et al., 1984). Fig. 2A shows that anti-CD3 antibody treatment (lane 2) resulted in an extensive DNA ladder formation compared to the untreated cells (lane 1), as expected. Percentage of apoptotic cells was also estimated by

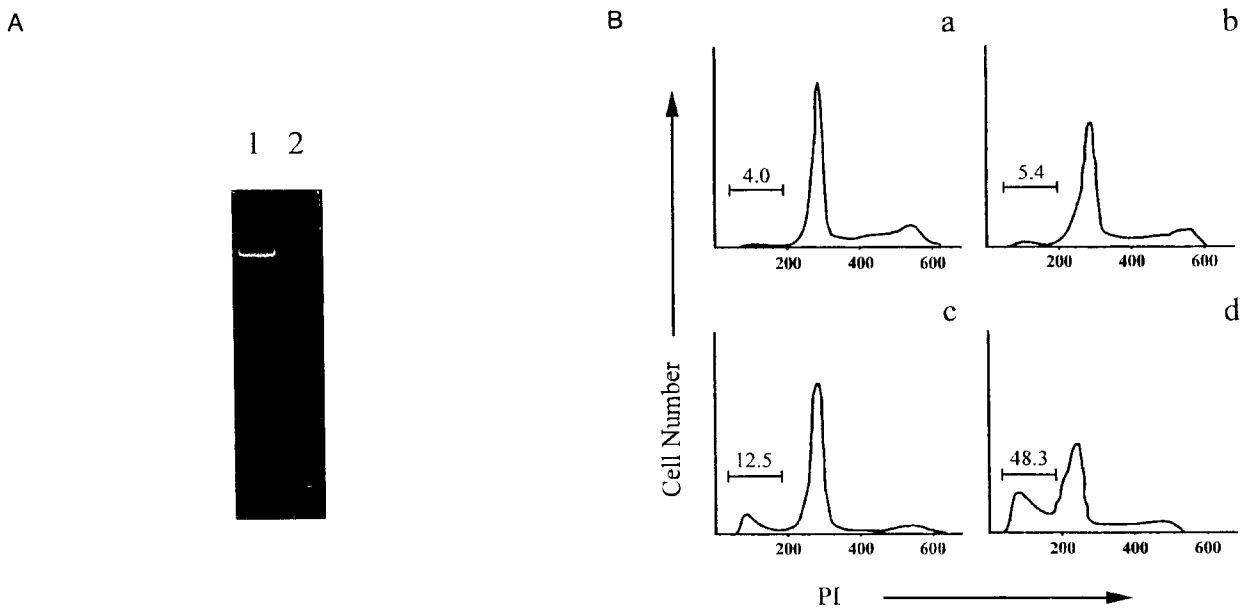

Fig. 2. Induction of apoptosis of T cell hybridoma. A, The T cell hybridomas, 70.7 were cultured on the 6 well plates coated with anti-CD3 antibody for 3 d. After induction of apoptosis, genomic DNA was analyzed by gel electrophoresis. Lanes 1, no treatment; 2, anti-CD3 antibody treated cells. B, FACS analysis of apoptosis induced cells. Cells were treated with 1 μM A23187 for 24 h (c) to 48 h (d) and stained with 50 μg/ml PI solution, and then their DNA contents were analyzed using FACStar. Untreated (a) and ethanol treated cells (d) were used as controls.

measuring DNA contents in the cells with propidium iodide (PI) staining followed by flow cytometry analysis (Fig. 2B). In this particular experiment, apoptosis was induced by calcium ionophore, A23187, which is known to increase intracellular calcium level and cause apoptosis of the T cells, as anti-CD3 antibody treatment does. Compared to the untreated and ethanol treated controls (Fig. 2B, a and b), the cells treated with A23187 (Fig. 2B, c and d) have the subdiploid amount of DNA, which represents the population of cells undergoing apoptosis. It also shows that the percentage of the apoptotic cells is increasing as fanction of time. These results clearly demonstrate that either the anti-CD3 antibody or calcium-ionophore treatment induces the T cell hybridoma to commit apoptotic cell death.

Next, we tested whether the apoptotic stimulus has any effect on the expression of *Tcf*-1 mRNA. Fig. 3A demonstrates that the expression of *Tcf*-1 mRNA was dramatically reduced when the T cell hybridoma was induced to apoptose by anti-CD3 antibody treatment (lane 2). To test whether it is due to the non-specific degradation of mRNA in the apoptotic cell, we examined the expression of other mRNAs by re-probing the same membrane with other probes, including *β-actin* and TBP (TATA-binding protein). As shown in the histogram of Fig. 3B, we could not observe any significant reduction of *β-actin* mRNA. Interestingly, the *TBP* mRNA level was shown to be increased to about 3-fold after apoptosis induction. In addition, a time-course experiment suggests that the reduction of *Tcf*-1 mRNA occurs at an early time point during the apoptotic process (data not shown). Thus, it is unlikely that the non-specific degradation of various mRNAs in the late stage of apoptosis is responsible for the observed reduction of *Tcf*-1 mRNA. Based on these experiments, we concluded that the *Tcf*-1 mRNA level is specifically reduced in apoptotic cells.

Down-regulation of CD3-ε following apoptosis induction

Tcf-1 is known to bind to the enhancers of various T cell specific genes, including CD3-ε and TCR-α genes; therefore, it would be interesting to test if the reduced expression of *Tcf*-1 following apoptotic stimuli would affect the expression of its target genes in these cells. We used CD3-ε as a model for *Tcf*-1 target genes to test whether these expressions were also reduced due to apoptosis induction. Because the CD3-ε protein is a component of the multiprotein complex of TCR/CD3 in the T cell surface, it is especially important to note that the down-regulation of CD3-ε expression would eventually reduce the number of TCR/CD3 complexes in the cell membrane. Previously, we have found that the expression of *CD3-ε* mRNA in apoptotic cells was significantly reduced (Jeon et al., 1998). Such a lower level of *CD3-ε* mRNA would lead to the lower amount of CD3-ε protein in apoptotic T cells. To measure the level of CD3-ε protein in apoptotic cells, cells were stained with the monoclonal antibody against the CD3-ε protein and analyzed by flow cytometry (Fig. 4). The profile of DNA content measured by PI staining showed a large proportion of the A23187 treated cells undergoing apoptosis (region 2). Cells in region 2 in Fig. 4A showed two major populations stained differently

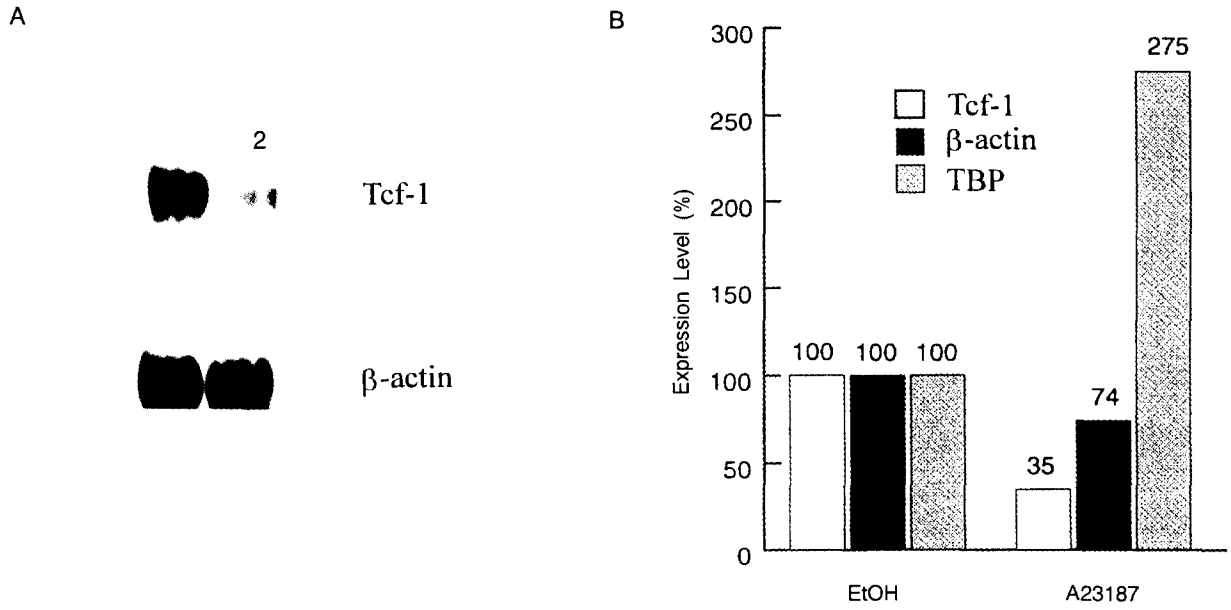

Fig. 3. Expression of the *Tcf-1* gene in apoptotic T cell hybridomas. A, Expression of *Tcf-1* in normal (lane 1) and apoptotic cells (lane 2). After treatment with anti-CD3 antibody for 3 d, the total RNA of 70.7 cells was isolated and analyzed by Northern blot analysis using the ^{32}P-labeled 1.3 Kb XhoI fragment of the *Tcf-1* gene as a probe. B, A histogram of the expression of various RNAs in apoptotic cells by A23187 treatment. Quantitated results were obtained from three independent experiments of Northern blot analysis.

with the CD3 antibody. The right peak represents the staining of both surface and cytosolic CD3-ε proteins expressed in a cell. The left peak represents only the staining of surface proteins. This becomes more clear when the sequence of fixation and antibody staining is reversed (Fig. 4B). When the cells were fixed with ethanol to make them permeable and then stained with the anti-CD3 antibody (F→S), the left peak representing only the surface staining shifted to the right peak. Therefore, cells in the right peak seemed to be dead and became permeable. When cells in the diploid peak (region 1 in Fig. 4A) were analyzed for the expression of CD3, most cells stained for the surface proteins and only a minor population of cells stained for the cytoplasmic proteins (Fig. 4C). On the other hand, when cells in the subdiploid region were analyzed for the expression of CD3, about 70% of the population stained both for the surface and cytoplasmic proteins and about 30% of cells stained only for the surface protein. Therefore, the cells staining only for the surface proteins seem to be the one involved in the apoptotic process, but not completely dead yet. Interestingly, these cells express a lower level (about 59% of the normal level) of surface TCR/CD3 complex compared to the normal ones with a diploid amount of DNA (region 1 in Fig. 4C).

These data clearly demonstrate that the cells undergoing apoptosis have lower levels of *CD3-ε* mRNA and surface proteins compared to normal cells. Because the CD3-ε protein is a component of the

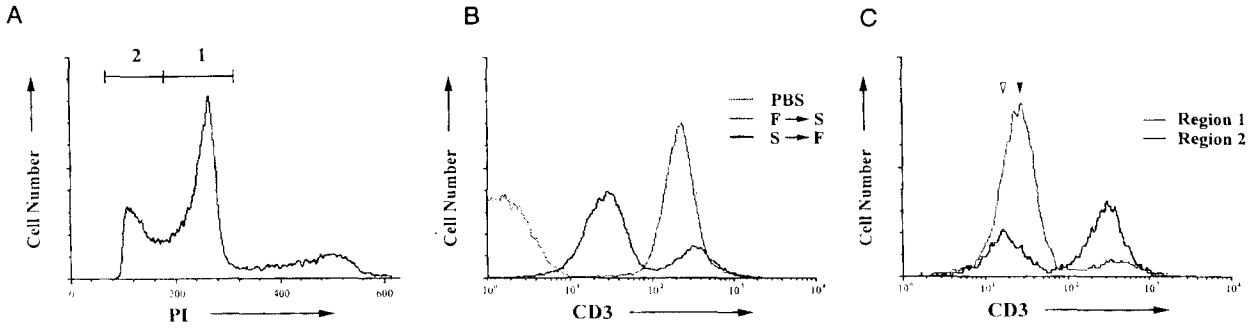

Fig. 4. Down-regulation of the CD3 expression in apoptotic T cell hybridomas. A, Cell cycle analysis of the 70.7 cells. Total cells were stained with FITC-conjugated anti-CD3 mAb, 145-2C11 prior to (S→F) or after (F→S) ethanol fixation, and stained with PI. B, In addition to the normal cells with a diploid amount of DNA (region 1), cells with a sub-diploid amount of DNA are also shown (region 2). The expression of the CD3 protein on the surface of apoptotic T-cells was down-regulated. C, The closed (region 1 in A) and open (region 2 in A) arrowheads indicate the peak of the graph. About 59% of surface TCR/CD3 complex was expressed on the cells with a sub-diploid amount of DNA content compared to the normal ones with a diploid amount.

multiprotein complex of TCR/CD3 in T cell surface, it is also expected that the down-regulation of CD3-ε protein would reduce the TCR/CD3 complex in the cell surface. Such a down-modulation severely impairs the signal transduction through the TCR/CD3 complex, thus it would affect the nature of T cells, possibly reducing the sensitivity to an antigen.

Discussion

We have cloned the *Tcf*-1 gene by subtractive hybridization and found that it is highly expressed in the thymus, but not in the spleen. We have also observed that, following apoptosic induction, the expression of *Tcf*-1 mRNA and one of its target gene, CD3-ε chain of TCR/CD3 complex, were specifically reduced. Such a down-regulation of CD3 would result in the reduction of surface TCR/CD3 complex on the apoptotic cell membrane. It would be important to explain the possible role of surface TCR/CD3 in apoptosis of T cells, however, its consequences and the exact mechanism of the down-modulation need to be understood by further studies.

We used T hybridoma cell line 70.7, as an *in vitro* model of T cells in this study. T cell hybridoma has been a valuable model system to study T cell apoptosis because it undergoes apoptosis after activation by various stimuli, including antibodies against the TCR/CD3 complex, glucocorticoids or a combination of phorbol ester and calcium ionophore (McConkey et al., 1989; Iwata et al., 1991; Jondal et al., 1995). We used anti-CD3 antibody treatment, which activates T cells by TCR/CD3 complex engagement followed by apoptosis of the cell. Such a treatment leads to an activation-induced apoptosis, which is known to operate in the negative selection of the thymus and in the deletion of activated T cells in the periphery. *De novo* synthesis of RNA and proteins are suggested for this process; Fas and Fas ligand are shown to be the death-gene products involved in the activation-induced T cell death, especially in clonal deletion or tolerance in the periphery (Osborne and Schwartz, 1994; Brunner et al., 1995; Cleveland and Ihle, 1995). Several genes have been implicated for the apoptosis, but the exact mechanism of these genes has just started to be understood in recent years (Nagata, 1997; Peter et al., 1997). Therefore, our finding that the *Tcf*-1 gene is regulated by apoptosis induction could give one clue as to how the apoptotic signal is executed by specific transcription factor in the signal pathway.

It is still not known how *Tcf*-1 acts as a transcriptional regulator and what the partners of *Tcf*-1 in the multiprotein complex in the T cells are. However, exciting new findings are emerging in other fields which can partly explain the mechanism of *Tcf*-1 activities (Clevers and van de Wetering, 1997). In oncogenic transformation of colon cancer cells and developmental pattern formation of Drosophila and Xenopus, it was clearly shown that *Tcf/Lef* protein is complexed with a β-catenin multifunctional protein and leads to the activation of the cells (Korinek et al., 1997; Morin et al., 1997; Riese et al., 1997). Even though the target genes of the *Tcf*/β-catenin complex are not known in these cells, it can be assumed that it might act as an activator of cell proliferation and/or as a suppressor of cell death. Thus, it would be interesting to note our finding that *Tcf*-1 is down-regulated during apoptosis, which probably worked as a suppressor of apoptosis. Whatever the biochemical mechanism of *Tcf*-1, its activity seems to be important for T cell differentiation and activation. Northern blot analysis and *Tcf*-1 knockout experiments suggest that *Tcf*-1 might play a role in early T cell development (Verbeek et al., 1995; Okamura et al., 1998).

Our finding that the *Tcf*-1 gene is specifically down-regulated following apoptosis induction is an important one in a few respects. First, *in vitro* expression of *Tcf*-1 mRNA can be partly explained in relation to its *in vivo* expression pattern. It is, in fact, known that *Tcf*-1 expression is tightly regulated during thymocyte differentiation when apoptosis plays a major role (Verbeek et al., 1995). Secondly, since the *Tcf*-1 transcription factor is a key regulator of T cell specific genes, especially TCR-α and CD3-ε, it is expected that the reduction of *Tcf*-1 expression would lower the amount of these target genes. This proposition has been tested in this study and found to be true for the CD3-ε protein. Such a down-regulation of CD3-ε and TCR would cause great reduction of surface TCR/CD3 complex. Third and most important result of this study is its implication in activation-induced apoptosis of the T cell. It is known that activation induced apoptosis of the T cell involves the regulation of TCR/CD3 complex on the cell surface. The level of surface TCR is a critical parameter in driving T cell activation and T cells become unresponsive or anergic to an antigen by down-regulating the surface level of TCR. It has been known that surface TCR is down-modulated by activating T-cells due to the internalization of the receptor complex (Telerman et al., 1987). The ligand-induced TCR/CD3 complex internalization was shown to happen in a relatively early stage of T-cell activation and to be mediated by endocytosis via clathrin coated pits (Minami et al., 1987; Dietrich et al., 1994). However, we have shown that the reduced expression of the surface TCR/CD3 during apoptosis is due to the reduced expression of *Tcf*-1, a major regulator for the transcription of *CD3-ε*. Therefore, the down-regulated expression of TCR/CD3 in apoptotic T-cells is due to a different mechanism from the one observed during the activation process. Such a down-modulation may severely reduce the signal transduction through the TCR/CD3 complex after interaction with a limited amount of antigen.

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