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Transcription Factor Activation during Signal-induced Apoptosis of Immature CD4$^+$CD8$^+$ Thymocytes

A PROTECTIVE ROLE OF c-Fos*

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Vladimir N. Ivanov and Janko Nikolić-Žugić‡

From the Immunology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Many signals that cause apoptotic cell death operate by inducing transcription and translation of other (presumably death effector) mediators, and it is well established that stimulus-induced apoptosis can often be blocked by inhibiting transcription and translation. Transcriptional regulation of apoptosis, however, is incompletely understood. To gain insight into nuclear events associated with signal-induced apoptosis during T cell development, we studied signal-induced apoptosis of *ex vivo* isolated immature CD8$^+$4$^+$ double-positive (DP) thymocytes. Stimuli utilizing the T cell receptor (TCR) signaling pathway or its parts (an αCD3/TCR monoclonal antibody, a Ca^{2+} ionophore, or a protein kinase C-activating phorbol ester) or a stimulus that antagonizes TCR signaling and apoptosis in T cell hybridoma (forskolin, a cyclic AMP-signaling activator) resulted in massive apoptosis of DP thymocytes. At the same time, these stimuli induced qualitatively similar but quantitatively unique patterns of inducible transcription factors (TFs) NF-κB/RelA-p50, AP-1 (Fos-Jun), and NUR-77. We focused our attention on the role of AP-1 (Fos-Jun) complex, which was strongly induced by all of the above stimuli and thus was a candidate for a proapoptotic TF. However, we found that AP-1/c-Fos induction was vital in prolonging DP thymocyte life, as judged by increased spontaneous and induced death of DP cells in Fos$^{-/-}$ mice. In direct support of this hypothesis, experiments with antisense oligonucleotides demonstrated that c-Fos plays an essential role in protecting normal DP thymocytes from Ca^{2+}- and cAMP-induced apoptosis but not from TCR-mediated death. Together, these results demonstrate a physiological role for c-Fos in maintaining longevity of DP thymocytes.

Apoptosis plays a key role in tissue modeling during normal development, yet many of its features remain obscure. One such feature is the transcriptional control of apoptosis. Signal-induced apoptosis is an active process that by definition requires transcription and translation (1). Such is the case in mature T cells, where a primary signaling cascade (*e.g.* the one initiated via the TCR[1] in T cells) would activate transcription of the genes whose products function in a secondary, "death effector" signaling, many of which belong to the TNF family (TNF, FasL, CD30L, etc.) (2). There is, however, a paucity of information concerning nuclear (transcriptional) changes that occur during initial phases of signal-induced apoptosis in other systems, and it is not clear whether the rule of primary-secondary cascades applies as well.

One of the most investigated models of apoptosis is the one using rodent thymocytes. In the steady-state adult murine thymus, apoptosis daily eliminates up to a third of all thymocytes, or up to 95% of all newly generated cells. The eliminated cells either failed positive selection (nonselected, or neglected, cells), or were triggered to die to prevent autoimmunity, because they bear potentially autoreactive receptors (negative selection) (3–5). The former type of death corresponds to "programmed" cell death, because the cells subjected to it, the CD8$^+$4$^+$ double-positive (DP) thymocytes, have a strictly limited life span of 2–3 days in the absence of positive selection (6). The latter type of death is induced by extracellular signals that chiefly operate via the TCR and is reminiscent of the activation-induced cell death (AICD) of mature peripheral T cells following exposure to antigen (while we shall use the term AICD for signal-induced apoptosis of thymocytes, it is important to bear in mind that the two phenomena are by no means identical). Experimental apoptosis of thymocytes can be readily induced *in vitro* by a variety of stimuli, which utilize different signaling pathways. Such stimuli include glucocorticoids (glucocorticoid nuclear receptor pathway), ionomycin (an activator of the Ca^{2+} pathway), PMA (an activator of protein kinase C and the Ras-Raf pathway), forskolin or prostaglandin E_2 (cAMP-dependent signaling), FasR/CD95 signaling, and γ-irradiation (7–13). Furthermore, negative selection of thymocytes can be mimicked *in vivo* and *in vitro* by agonistic αCD3/TCR-specific mAbs (10, 14, 15), which, similarly to the natural TCR ligands, elicit stimulation of the downstream Ca^{2+}, protein kinase C, and other pathways. The bulk of thymocyte apoptosis in these experimental systems and in the course of *in vivo* thymocyte selection (16) occurs among the CD4$^+$CD8$^+$ DP thymocytes, precisely because they are the population undergoing selection. However, relative roles and the interplay of distinct signaling pathways during physiological development and selection of thymocytes are poorly understood, as is the role of transcriptional control of the above processes.

To understand the molecular basis of AICD in thymocytes, we investigated transcription factor (TF) induction and apoptosis in DP thymocytes in response to a variety of stimuli

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‡ To whom correspondence should be addressed: Memorial Sloan-Kettering Cancer Center, Box 98, 1275 York Ave., New York, NY 10021. Tel.: 212-639-2387; Fax: 212-794-4019; E-mail: nikolicj@mskcc.org.

[1] The abbreviations used are: TCR, T cell receptor; DP, double-positive; AICD, activation-induced cell death; AP-1, activator protein-1; Ab, antibody; mAb, monoclonal antibody; TF, transcription factor; CREB, cyclic AMP-response element-binding protein; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor κB; PI, propidium iodide; PMA, phorbol 12-myristate 13-acetate; MOPS, 4-morpholinepropanesulfonic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF FCM-flow cytometry, tumor necrosis factor α.

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connected to TCR signaling. We have chosen to follow the inducible transcription factors of the NF-κB/Rel family (17), AP-1 (18), NUR-77 (19, 20), and CREB/ATF (21), because their DNA binding activity was shown to be regulated by signals inducing cell activation and death. NF-Y (22) was used as a reference factor with stable DNA binding activity. We describe a complex pattern of transcription factor induction and provide evidence for an antiapoptotic role for c-Fos.

EXPERIMENTAL PROCEDURES

Mice—Female C57BL/6 (B6), B6*fos*$^{-/-}$ (Jackson Laboratory, Bar Harbor, ME), and Bcl-2 transgenic mice on B6 background (Ref. 23; generously provided by Dr. S. Cory, WEHI, Melbourne, Australia, via Dr. H. T. Petrie, Memorial Sloan-Kettering Cancer Center, New York) were used at 4–8 weeks of age.

Thymocyte Preparation, Activation, and Flow Cytometry (FCM)—$CD4^+CD8^+$ DP thymocytes were enriched from total thymocytes by panning with anti-CD8 mAb (3.155), immobilized on the surface of plastic dishes, as described previously (24). Alternatively, these cells and control $CD8^+CD4^-$ were sorted to >99% purity following staining with directly conjugated anti-CD8 and anti-CD4 mAbs using a FACStar Plus (Becton Dickinson, Mountain View, CA).

$CD4^+CD8^+$ thymocytes were activated with 10 μg/ml immobilized anti-CD3ε (145–2C11) or soluble anti-Fas mAb (Jo2) (both from PharMingen, San Diego, CA) in 24-well flat-bottom plates. Forskolin (10 μM), ionomycin (250–1000 ng/ml), PMA (10 ng/ml), dexamethasone (1 μM), cycloheximide (10 μg/ml), and actinomycin D (200 ng/ml) were purchased from Sigma. Thymocytes were directly stained with excess FITC-anti-CD8, PE-anti-CD4 (Becton Dickinson, Mountain View, CA) and FITC-anti-TCRβ. FCM analysis was performed on a FACScan flow cytometer using Lysis II software (Becton Dickinson).

DNA Fragmentation Analysis—This assay was performed as described previously (25). Cells were pelleted and resuspended in 0.5 ml of hypotonic buffer with 0.1% Triton X-100 containing propidium iodide (PI) (40 μg/ml) and DNase-free RNase A (10 mg/ml). Cells were incubated at 37 °C for 30 min and analyzed by FCM on a FACScan (Becton Dickinson, CA). The percentage of cells to the left of the diploid $G_{0/1}$ peak, diagnostic of hypodiploid cells that have lost DNA, was taken as the percentage of apoptotic cells. Cell viability was also scored by trypan blue and PI exclusion and was concordant with the degree of apoptosis.

Oligonucleotides and Electrophoretic Mobility Shift Assay (EMSA)—The following double-stranded oligonucleotides were used in this study as specific probes for transcription factors (only one strand of a double-stranded oligonucleotide is shown; binding sites of transcription factors are underlined; lowercase nucleotides denote ends introduced for unrelated cloning purpose): agctTGGGGACTTTCCAGCCG for NF-κB; agctTGATGAGTCAGCCG for AP-1; agctCCATGACGTCATGG for CREB; GGAGTTTTAAAAGGTCATGCTCAATTT for NUR-77; GTCTGAAACATTTTTCTGATTGGTTAAAAGTTGAGTGCT for NF-Y.

Oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Nuclear extracts were prepared from 20×10^6 thymocytes unless indicated otherwise. Binding reactions were carried out by incubating the end-labeled DNA (50,000 cpm) with 2 μg of nuclear proteins and 2 μg of poly(dI-dC), as described previously (26). For identification of transcription factors, nuclear extracts were preincubated with 1 μl of specific antiserum for 15 min at 20 °C before the addition of the labeled oligonucleotide probes. The incubation was continued for another 30 min and followed by EMSA. Polyclonal rabbit antisera to the p50 subunit of NF-κB (27), to an N-terminal peptide of the RelA/p65 subunit of NF-κB (28), to a C-terminal peptide of c-Rel (29), to mouse RelB, JunB, and JunD (30, 31), and to the full-length mouse c-Fos (32) and an antiserum to v-Jun that cross-reacts to c-Jun (33) were kindly provided by Drs. M. Lenardo (NIH, Bethesda, MD), W. Greene (Gladstone Institute, San Francisco, CA), N. Rice (NCI, Frederick, MD), R. Bravo (Bristol-Myers, Princeton, NJ), T. Curran (Roche Institute of Molecular Biology, Nutley, NJ) and H. Rahmsdorf (University of Karlsruhe, Germany). Quantification of the band intensity in the EMSA assay was performed using the Bio-Rad molecular imaging system (model GS-250), equipped with Phosphor Analyst software (Bio-Rad), and is expressed relative to the intensity of bands without stimulation, normalized to the levels of the reference TF, NF-Y.

Northern and Western Analysis—Cytoplasmic cell extracts were used for isolation and purification of total RNA by the SDS-phenol method. Total RNA was separated on formaldehyde/MOPS-agarose gel and blotted on Hybond N membranes (Amersham Corp.), which were hybridized with ^{32}P-labeled DNA probes for GAPDH, Jun-D (34) and c-Fos (32). Induction of transcription factor mRNA was quantified using the Bio-Rad molecular imaging system (model GS-250), equipped with Phosphor Analyst software (Bio-Rad) and is expressed relative to the level of the reference mRNA for the housekeeping gene, GAPDH.

For Western blot analysis, nuclear proteins were resolved by 10% SDS-PAGE, transferred to nitrocellulose and processed by a standard protocol provided in the manufacturer's manual (Amersham). Polyclonal anti-c-Fos antibody was used at 1:1000. Signals were detected using ECL (Amersham) and were quantified by densitometry.

Antisense Oligonucleotide Treatment—The sense and antisense phosphorothioate analogues of the oligonucleotides to the 5′-end of c-Fos mRNA including the ATG initiation codon were synthesized (c-Fos sense oligonucleotide, TCG ACC ATG ATG TTC TCG GGT; c-Fos antisense oligonucleotide, ACC CGA GAA CAT CAT GCT CCA (35)). Rel-A sense oligonucleotide (CTG ACC ATG GAC GAT CTG TTT CCC) was used as another negative control. Purified oligonucleotides were obtained from the Memorial Sloan-Kettering Cancer Center Microchemistry Core Facility and were used at indicated concentrations. For all experiments, cells were preincubated for 30 min with oligonucleotides, followed by stimulation with activators for an additional 16 h. Apoptosis was determined as described.

RESULTS

Signal-induced Apoptosis in DP Thymocytes—To investigate TF involvement in thymocyte apoptosis, we first established a model of cortical thymocyte apoptosis. We enriched $CD8^+4^+$ cells to 91 ± 2% from total thymocyte suspensions, using panning with immobilized anti-CD8 mAb (Fig. 1*A*). The purity of DP cells could be further increased to 94–96% by the second round of panning using immobilized anti-CD4 mAb, but such a treatment, even at 4 °C, had a strong inhibitory effect on transcription factor induction (not shown), reminiscent of its effect on mature T cell activation (36). Inasmuch as our final goal was to investigate the relationship between TF induction and AICD, we avoided extensive CD4 cross-linking and used anti-CD8 panning in most studies.

As stimuli for AICD, we used full or partial agonists of TCR signaling (αCD3 mAb, PMA, and ionomycin) and an antagonist of TCR signaling in T cell hybridomas (forskolin, a cAMP signaling activator). As a positive control, we used dexamethasone, an apoptosis-inducing stimulus that operates via the glucocorticoid nuclear receptor and is not connected directly to TCR signaling. All of the above stimuli induced extensive apoptosis in DP thymocytes following overnight treatment, as determined by quantifying cells with hypodiploid DNA content, diagnostic for apoptosis (Fig. 1*B*, *lower panels*). Cell counting using trypan blue (optical microscope) or propidium iodide (FCM) exclusion, confirmed that cell loss was substantial in each case where the numbers of hypodiploid cells were increased, while that was not the case when few hypodiploid cells were observed (not shown).

The aim of this work was to investigate whether this apoptosis may be linked to a specific pattern of TF induction. It was, therefore, important to confirm that all apoptosis in the above models was indeed due to new transcription and translation. To that effect, we stimulated DP thymocytes in the presence of transcription and translation inhibitors actinomycin D (200 ng/ml, not shown) and cycloheximide (10 μg/ml, Fig. 1*B*, *upper traces*). As shown in numerous other studies (1, 7–11), we found that apoptosis induced by TCR agonists, forskolin, and dexamethasone was inhibited by >90% by actinomycin D or cycloheximide and thus was dependent on new transcription and translation (Fig. 1*B*). By contrast, FasR-mediated apoptosis (induced by an agonistic mAb Jo2; Ref. 16) did not require transcription and translation, consistent with its rapid kinetics and its proteolytic mechanism of apoptosis induction (not shown).

By itself, forskolin induced apoptosis in DP thymocytes in a dose-dependent manner (Fig. 1*B* and data not shown). Since forskolin is known to antagonize TCR signaling in hybridomas

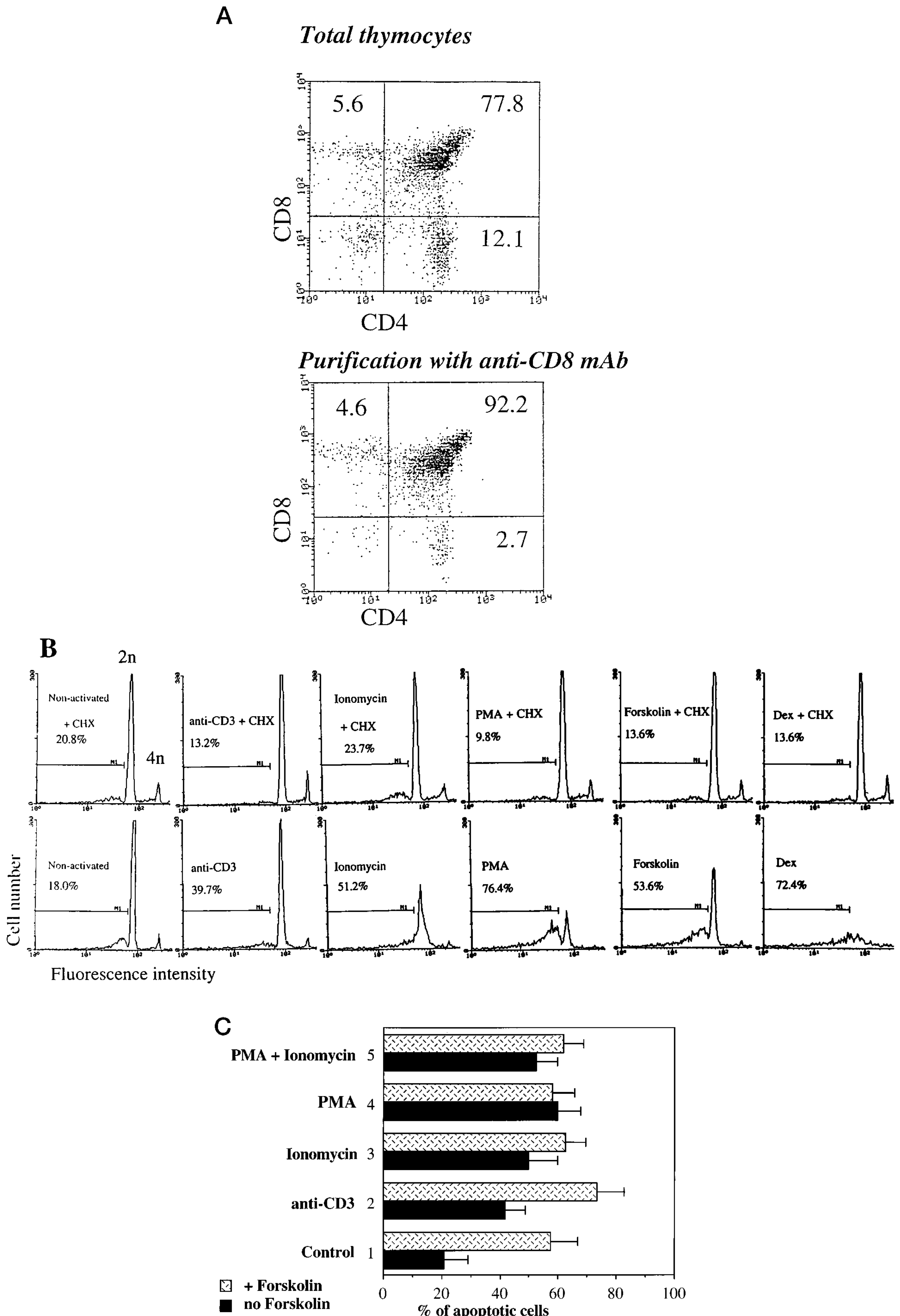


FIG. 1. **AICD in enriched CD4⁺CD8⁺ (DP) thymocytes.** *A*, enrichment of DP thymocytes by panning using immobilized anti-CD8 mAbs. Positively selected thymocytes were directly stained with FITC-anti-CD8 and PE-anti-CD4 mAbs and analyzed by FCM. *B*, determination of apoptosis levels by PI staining. DP-enriched thymocytes were treated for 16 h with no stimulus, immobilized anti-CD3ε mAb, ionomycin (250 ng/ml), PMA (10 ng/ml), forskolin (10 μM), and dexamethasone (*Dex*; 1 μM) in the absence (*lower traces*) or the presence (*upper traces*) of 10 μg of cycloheximide (*CHX*) and assayed for DNA fragmentation by PI staining as described under "Experimental Procedures." The marker was set to

(38), we sought to examine whether forskolin may also affect DP apoptosis regulated by other stimuli. 10 μM forskolin did not affect PMA- or ionomycin-induced cell death, and, surprisingly, it appeared to synergize with TCR-mediated death of DP thymocytes (Fig. 1*C*). These results stand in striking contrast with the inhibitory effect of forskolin on TCR-dependent apoptosis of a T cell hybridoma (38).[2] Thus, the response of T cell hybridomas, which were routinely used as models of thymocyte apoptosis, was much closer to the response of mature T cells.

Signal-dependent Activation of Transcription Factors in DP Thymocytes—We next investigated whether the above stimuli induced a discernible pattern of TF activation and whether any of the putative changes in TF activity may be causally related to AICD of DP thymocytes. For this study, we selected TF AP-1, NF-κB, and CREB, which are strongly induced in the course of T cell activation, and the orphan steroid receptor and a putative TF NUR-77, which is not only induced following activation but also plays a role in apoptosis (19, 20). As a control, we used a conserved and constitutively expressed TF NF-Y (22). DP thymocytes were stimulated for 3 h with the stimuli studied in Fig. 1, and nuclear extracts from stimulated cells were used to evaluate the induction of indicated TF by EMSA (Fig. 2*A*). PMA (10 ng/ml), PMA plus ionomycin (250 ng/ml), and anti-CD3ϵ strongly up-regulated the upper NF-κB DNA-binding complex 6–8-, 4-, and 4–5-fold, respectively (as determined by densitometry) and increased the level of the lower complex uniformly by about 2-fold. Ionomycin similarly increased the lower complex (3-fold), but its effects on the upper complex were more variable: in some experiments (Fig. 2*A*), this agent only weakly up-regulated the upper NF-κB complex (1.5-fold), while in others the up-regulation of the upper complex was more vigorous (data not shown). This is consistent with pleiotropic effects of Ca^{2+} on gene expression and suggests that other, presently unknown, factors may modulate the effects of Ca^{2+} on transcription factor induction. All of the above stimuli also induced the AP-1 activity; the upper band was induced 2.5-fold by PMA, 3-fold by ionomycin, 6-fold by their combination, and 3–3.5-fold by αCD3 (Fig. 2*A*). NUR-77 DNA binding activity was induced preferentially by Ca^{2+}-inducing stimuli (ionomycin, ionomycin plus PMA, or anti-CD3, all in the range of 2–3-fold) but not by PMA alone. Levels and the appearance of CREB/ATF did not vary very much for ionomycin but were up-regulated by αCD3 and forskolin (data not shown, and Figs. 2*B* and 3). This, taken together with the phenotype of the mouse carrying a dominant negative mutant of CREB/ATF, which does not exhibit discernible defects in T cell development (39), suggested to us that CREB may not be universally involved in thymocyte apoptosis, although further experiments are necessary to directly examine this issue. As expected, the level of the reference transcription factor NF-Y was stable (<5% variation), irrespective of stimulation (Fig. 2*A*).

cAMP-mediated signaling (forskolin) was shown to induce apoptosis of immature thymocytes (Ref. 9; Fig. 1*C*). By contrast, cAMP activation antagonized TCR-dependent signaling in T cell hybridoma and inhibited activation-induced apoptosis of these cells (38, 40), and we therefore examined the effects of forskolin on TCR-induced TF activation in DP thymocytes. Forskolin stimulation of DP thymocytes up-regulated both nuclear NF-κB complexes, RelA-p50 and p50-p50, by 1.5–2-fold,

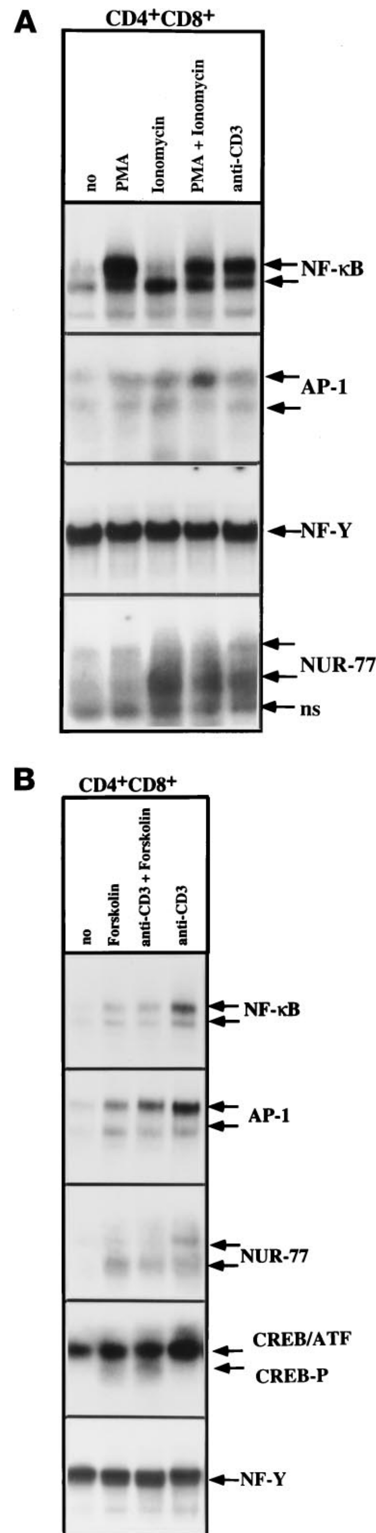


Fig. 2. **Transcription factor induction in DP thymocytes by AICD-promoting stimuli.** *A* and *B*, induction of TF by TCR-related signaling. EMSA was performed with nuclear extracts of DP thymocytes stimulated for 3 h with indicated stimuli, at doses detailed in Fig. 1, using labeled oligonucleotide probes for NF-κB, AP-1, NUR-77, CREB, and NF-Y transcription factors. Quantification of band intensity relative to basal levels was performed using imaging (see "Experimental Procedures").

[2] Ivanov, V. N., Lee, R. K., Podack, E. R., and Malek, T. R. (1997) *Oncogene* **14,** in press.

delimit the percentage of apoptotic cells, containing less than the diploid amount of DNA. *C*, effects of cAMP signaling on activation-induced cell death. DP thymocytes were activated as indicated for *B*, in the presence or absence of 10 μM forskolin. Results from at least five different experiments were used to obtain mean values ± S.D.

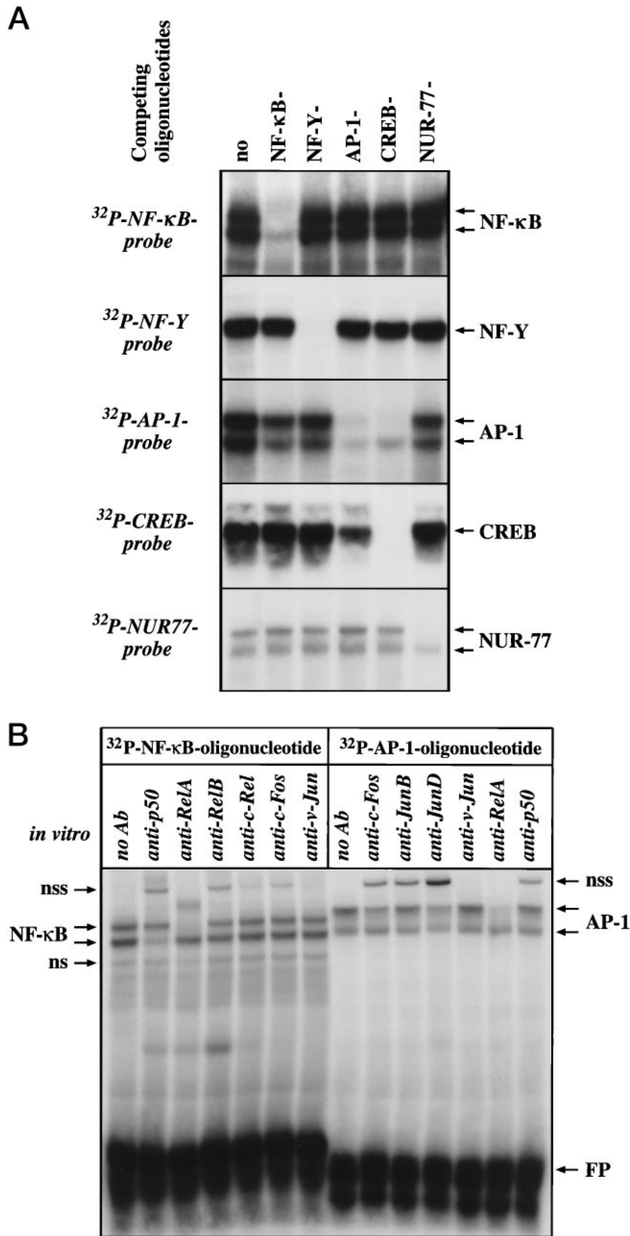


FIG. 3. **Identification of transcription factors induced in DP thymocytes following αCD3-stimulation.** *A*, competition with homologous and heterologous oligonucleotides. EMSA was performed with nuclear extracts of αCD3-stimulated DP thymocytes in the presence of a 250-fold excess of unlabeled oligonucleotides indicated *above* the *lanes*. *B*, identification of NF-κB and AP-1 DNA-binding complexes. Nuclear extracts from αCD3-treated DP cells were preincubated with antisera to NF-κB/RelA, RelB, c-Rel, p50, v-Jun/c-Jun, JunB, JunD, or c-Fos, as indicated *above* the *lanes*, before EMSA, using ^{32}P-κB (*left seven lanes*) or ^{32}P-AP-1 (*right seven lanes*) oligonucleotides. Nonspecific bands that could be observed after the interaction of labeled probes with antisera without nuclear extracts are designed *nss*. Band intensity was quantified relative to basal levels using imaging (see "Experimental Procedures").

as well as AP-1 and NUR-77 (2-fold each) after 3 h (Fig. 2*B*). Forskolin also induced the appearance of an additional diffuse band of CREB with increased mobility (probably a phosphorylated form of CREB), designed as CREB-P (Fig. 2*B*). By itself, αCD3 induced robust RelA-p50 activity (the upper complex was up-regulated 5-fold) and AP-1 upper complex (Fos-Jun) activity (3–3.5-fold induction). When administered with αCD3, forskolin decreased anti-CD3-induced levels of RelA-p50 to the levels induced by forskolin alone, although this effect was less pronounced than the one observed in a T cell hybridoma.[2] Likewise, forskolin partially suppressed TF binding activities of AP-1 and NUR-77 induced by αCD3 (Fig. 2*B*). Thus, although cAMP signaling partially down-regulated TF activities induced by anti-CD3, this down-regulation did not result in the suppression of anti-CD3-induced apoptosis of DP thymocytes. In fact, we observed additive effects of TCR-dependent and cAMP-dependent signaling in the induction of apoptosis (Fig. 1*C*). We conclude that transcriptional regulators different from NF-κB, NUR-77, and AP-1 are likely to be involved in TCR-mediated AICD of DP thymocytes but may not operate in mature T cells, as judged by the behavior of a T cell hybridoma (38, 40).

Enriched DP cells used for the above experiments still contained 8–10% contaminating cells of other phenotypes, the most abundant (up to 6%) being the $CD8^+4^-$ single-positive. To control for the effect of this contamination, we performed experiments using >99% pure DP and single-positive $CD8^+CD4^-$ thymocytes (FCM sorting) and showed that NF-κB, AP-1, and NUR-77 activities could be induced in sorted DP thymocytes but not in nuclear extracts of $CD8^+$ single-positive cells, when the two were used in the amounts (2 μg and 100 ng, respectively) representative of their ratios present in preparations obtained by panning (not shown). CREB, however, was present at relatively high levels following αCD3 induction. Since CREB is highly inducible in mature T cells, this observation can be explained by the maturational status of $CD8^+$ thymocytes, which are quite similar to their peripheral counterparts. These results exclude the role of contaminants in the observed TF induction, and demonstrate that thymocyte preparations obtained by panning closely approximate the characteristics of pure DP thymocytes. Therefore, all subsequent experiments were performed with DP thymocytes enriched by panning.

Identification of TF Induced by TCR Signaling—Competition experiments with the excess of cold homologous or heterologous oligonucleotides demonstrated the specificity of two NF-κB DNA-binding complexes, two AP-1 complexes, two NUR-77 complexes, CREB, and the NF-Y complex (Fig. 3*A*). Cross-inhibition was observed with the ^{32}P-AP-1 probe, whose interaction with nuclear extracts was inhibited not only by the specific cold oligonucleotide but also by the CREB-binding oligonucleotide (Fig. 3*A*). This, however, is not surprising, since the AP-1 and CREB binding sites share considerable homology and differ by only a single nucleotide. Interestingly, interactions of CREB with its specific site were less sensitive to such heterologous inhibition, although partial inhibition by the AP-1 oligonucleotide was observed (Fig. 3*A*).

Positive identification of DNA-binding complexes was achieved by pretreatment of nuclear proteins with Abs against specific transcription factors, followed by EMSA. Such treatment results in specific inhibition and/or supershifts of DNA-binding complexes. Results shown in Fig. 3*B*, using extracts from αCD3-stimulated cells, illustrate this type of analysis. As expected, results indicated that the upper NF-κB complex was mainly composed of the RelA and p50 (60–70% inhibition with anti-RelA and 23–25% with anti-p50), while the lower band contained the NF-κB p50-p50 homodimer (inhibited by >60% with anti-p50). Neither ionomycin nor forskolin induced nuclear RelB or c-Rel activity in DP thymocytes (not shown). By contrast, PMA (not shown) and anti-CD3 (Fig. 3*B*) induced high levels of RelA-p50 (>60% of the upper complex intensity in Fig. 3*B* was inhibited with αRelA Ab, as judged by densitometry) and low levels of RelB-p50 (20–25% inhibition by RelB Ab), which co-migrated as the upper band. No c-Rel activity was detected (<5% inhibition).

The upper AP-1 complex is canonically a heterodimer of Fos-Jun subunits (18), as was clearly shown by inhibiting this

DNA-binding complex with antibodies to Fos (40% inhibition), JunD (40% inhibition), and JunB (20%). Other Fos family members (FRA1 and -2) could also have been present. However, besides c-Fos, JunD, and JunB, the upper AP-1 complex induced in DP thymocytes surprisingly contained NF-κB RelA (Fig. 3*B*). This AP-1 complex did not contain any RelB or c-Rel activity and, at best, contained only very low amounts of c-Jun and NF-κB p50. We were able to identify the lower band of the AP-1 complex from DP thymocytes as a Jun-Jun combination, containing JunB and JunD (inhibited by specific antibodies by 20–30% and 40%, respectively), but again very little c-Jun (less than 5% inhibition) (Fig. 3*B*) (it should be noted that the band labeled *nss* denotes a nonspecific complex, which is a result of interaction of serum proteins and labeled probes, even in the absence of nuclear proteins). We conclude that stimulation of DP thymocytes, in addition to Fos-JunD and Fos-JunB complexes, induced a supercomplex of AP-1 and RelA in thymocytes, reminiscent of the one described recently in HeLa cells (41).

Effect of Antisense c-Fos Oligonucleotide Treatment on Induced Levels of Cell Death—All stimuli (ionomycin, PMA, a combination of PMA and ionomycin, forskolin, and anti-CD3) induced AP-1/c-Fos TF activity, although PMA alone was not as consistent and strong an inducer as the other stimuli (not shown). We next tested whether this induction occurs at the level of c-Fos mRNA. Northern blot analysis followed by PhosphorImager quantification (Fig. 4) showed that c-Fos mRNA was strongly up-regulated by cAMP and Ca^{2+} signaling but was at best very weakly induced by αCD3.[3] Therefore, transcriptional control of c-Fos expression was stimulus-specific.

To determine whether cell death may be dependent on inducible c-Fos expression and activation, we performed antisense inhibition of c-Fos translation in DP thymocytes, followed by stimulation of pretreated cells with forskolin, ionomycin, PMA, or dexamethasone (Fig. 5, *A* and *B*). Oligonucleotides at concentrations used in these experiments were not toxic for DP thymocytes, since they did not induce necrotic or apoptotic cell death (Fig. 5 and data not shown). c-Fos suppression by antisense oligonucleotide down-regulated the AP-1 binding activity (inhibition of the Fos-Jun band by 2.5–3-fold by ionomycin, and by 1.5–2-fold by forskolin; Fig. 5*C*), decreased the levels of Fos protein by 2–2.5-fold as judged by Western blot analysis (Fig. 5*D*), and specifically increased cell death levels induced by forskolin (in the oligonucleotide range 0.5–5 μg/ml) and ionomycin (1–10 μg/ml) but not by PMA or dexamethasone (Fig. 5*A*), consistent with the relatively weak induction of c-Fos by PMA and with the finding that dexamethasone negatively regulates AP-1-dependent transcription (42). The addition of sense Fos or sense RelA oligonucleotide had no significant effects on any of the three parameters examined (Fig. 5). The fact that c-Fos antisense treatment did not completely block all AP-1 activity and apoptosis likely reflects the activity of other Fos family members (FRA-1 and FRA-2), which can substitute for Fos in the upper AP-1 complex. These family members were shown to substitute for Fos in the Fos^{-} mice (49, 50). These results showed that in normal DP thymocytes c-Fos induction correlated with the protection against Ca^{2+} and cAMP-induced cell death.

The antiapoptotic protooncogene product Bcl-2 (43, 44) is developmentally regulated during T cell differentiation (45, 46) and was implicated in regulating thymocyte survival *in vivo* (47), but the mechanism of its action is still obscure. To investigate whether c-Fos may mediate the antiapoptotic effects of Bcl-2, we took advantage of Bcl-2 transgenic thymocytes (23). However, despite blocking c-Fos with antisense oligonucleotides, transgenic Bcl-2 still suppressed DP cell death (Fig. 5*B*),

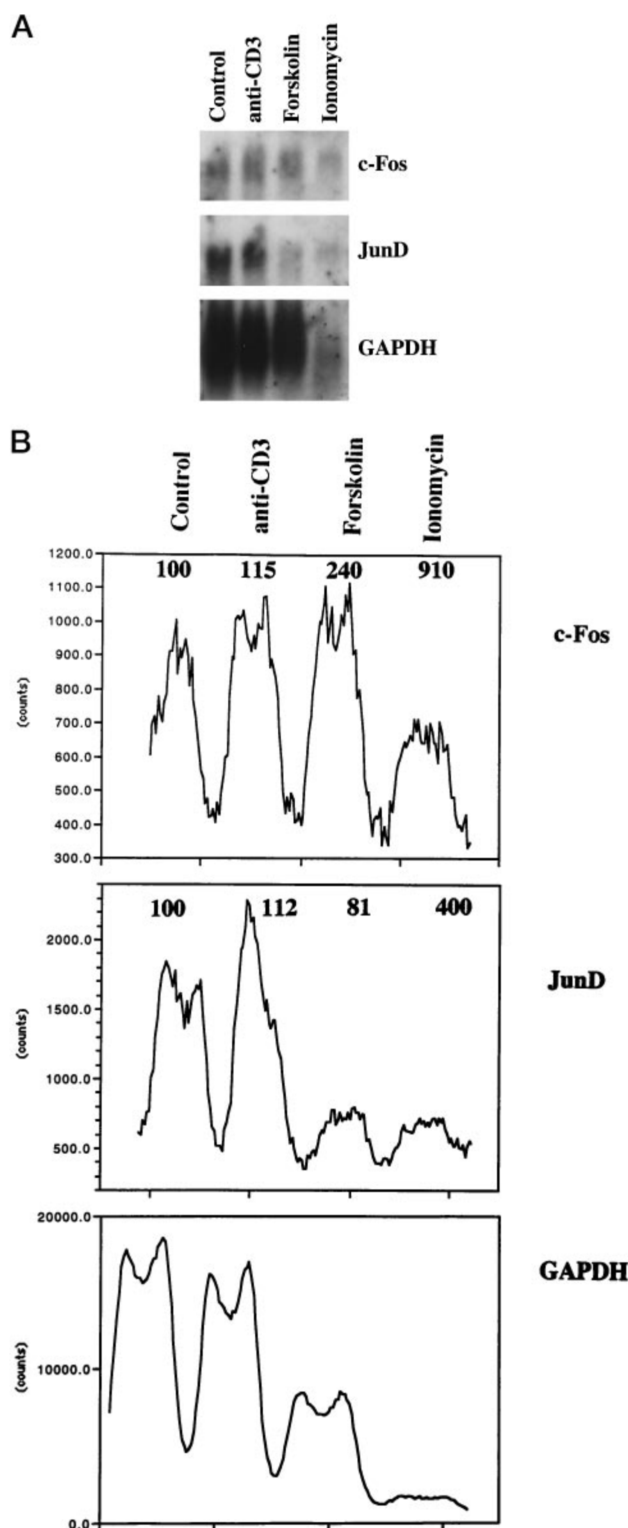
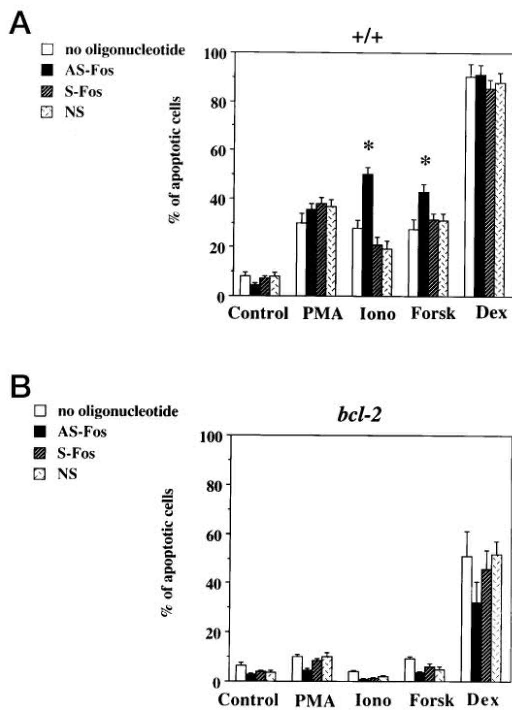


FIG. 4. **Quantification of the Northern hybridization analysis of c-Fos and JunD mRNA expression.** Two hours following treatment of DP thymocytes with indicated stimuli, total cellular RNA was isolated and probed as described under "Experimental Procedures" (*A*). Intensity of specific bands was quantified by scanning on a Molecular Imager system (*B*). Results are presented as percentage of intensity relative to the levels of a reference housekeeping mRNA, GAPDH.

[3] Note that the values given are calculated relative to the reference housekeeping gene GAPDH. Hence, although absolute levels of Fos induced by ionomycin are lower than following αCD3, most probably owing to strong activation of RNases following ionomycin treatment, its induction relative to GAPDH is much higher.



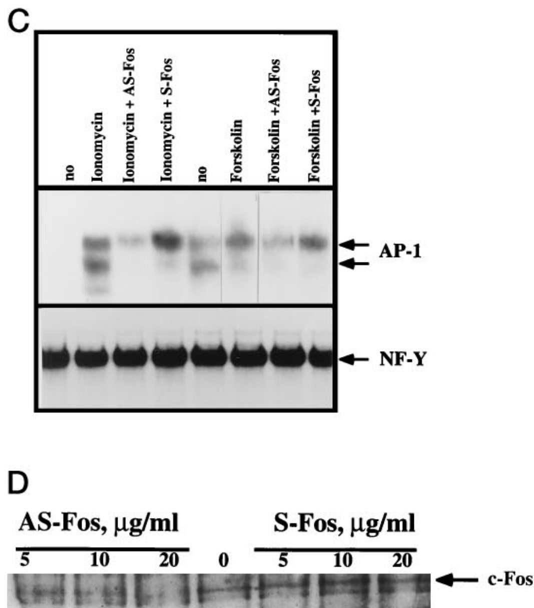


FIG. 5. **Effect of antisense c-Fos oligonucleotide on apoptotic death of DP thymocytes.** Cell death was induced by forskolin, ionomycin, PMA, or dexamethasone (*Dex*) (*A*) in normal and Bcl-2 transgenic (*B*) mice. DP thymocytes from normal or Bcl-2 transgenic mice were treated as indicated in the presence of different concentrations of antisense c-Fos- or sense c-Fos-oligonucleotides, and the representative results are shown for 10 μg/ml. 18 h after treatment, the percentage of apoptotic cells was determined by PI staining and flow cytometry. Significant differences occurring as a consequence of antisense treatment are indicated by the *asterisks*. *C*, control EMSA was performed with nuclear extracts isolated from DP thymocytes 3 h after treatment with indicated stimuli in the presence and absence of indicated oligonucleotides (10 μg/ml). Treatment by ionomycin or forskolin was performed in separate experiments, and basal levels of AP-1 were different for these experiments. *D*, Western blot analysis of Fos protein levels, using an anti-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Results from one experiment, representative of two others, are shown. Induction and inhibition of complexes for the EMSA assay were quantified as described for Figs. 2 and 3, while the quantification of Northern and Western analysis was performed as described under "Experimental Procedures."

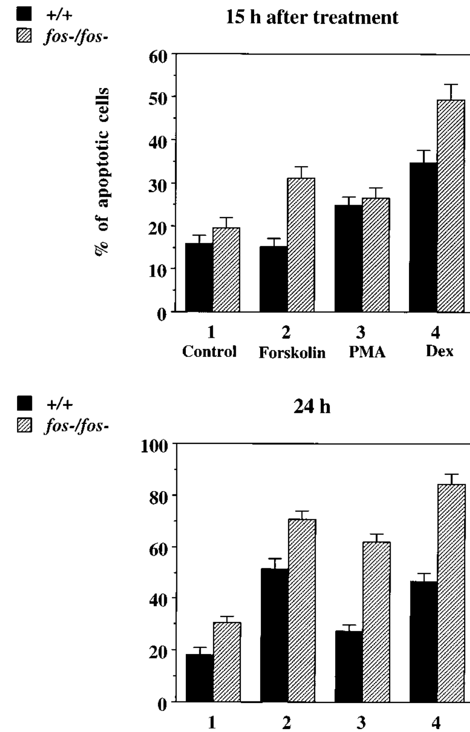


FIG. 6. ***fos$^{-/-}$* mice exhibit an increased sensitivity to spontaneous apoptosis and AICD.** DP thymocytes from three normal and three *fos$^{-/-}$* animals were pooled and treated as indicated and were assayed for apoptosis by FCM at two different time points. Results were confirmed in another experiment. *Dex*, dexamethasone.

indicating that c-Fos was not an essential mediator of Bcl-2-dependent protection against apoptosis.

DP Thymocytes from fos$^{-/-}$ Mice Are Hypersensitive to Apoptosis—*fos$^{-/-}$* mice exhibit a number of abnormalities, including prominent bone malformations and a hypoplastic lymphoid system (48, 49). Their thymocytes were reported to be severely depleted (48) or normal (50) by two different groups. To evaluate the propensity of *fos$^{-/-}$* DP thymocytes to undergo apoptosis, we stimulated them with forskolin, PMA, and dexamethasone. These experiments revealed that the absence of c-Fos leads to a general increase in susceptibility to both spontaneous and induced apoptosis (Fig. 6). Increase in spontaneous apoptosis, as well as the increase in apoptosis following stimulation with factors that do not (dexamethasone) or only slightly (PMA) induce c-Fos, suggest that basal levels of Fos play a role in protecting from apoptosis. Together, these results establish c-Fos as an anti-apoptotic factor in DP thymocytes.

DISCUSSION

The most important finding of this study is that, at physiological levels, c-Fos can play an important role in preventing apoptosis of DP thymocytes in response to Ca^{2+} and cAMP signaling. These results are supported by the recent elegant experiments with UV treatment of c-*fos$^{-/-}$* mouse fibroblasts, which also describe the role for c-Fos in inhibiting apoptosis (51). Previous observations with the basic phenotype of Fos knockout mice were initially controversial. One group reported no alteration of thymic size and weight, whereas the other reported a severe reduction in thymic size in adult (6-week) but not neonatal (2-week) animals (50, 48). Subsequent experiments revealed that thymic alterations occurred secondary to bone abnormalities, which then affected the bone marrow, since *fos$^{-/-}$* bone marrow cells developed normally into T and B cells when transferred into normal recipients (48). Overexpression of transgenic c-Fos also resulted in reduced thymocyte

numbers, most likely secondary to a deregulated proliferation of the thymic epithelium (52). However, spontaneous or stimulus-induced thymocyte apoptosis was never directly investigated in the above knockout and transgenic models. Our results demonstrate that *fos*$^{-/-}$ DP thymocytes do not survive as well as normal DP cells, providing further evidence for the role of c-Fos in positively regulating thymocyte survival. In two other nonlymphoid transformed cell systems, transfection of chimeric c-Fos was recently shown to induce cell death by apoptosis (53, 54). Two explanations can be put forward to reconcile these observations with our results and those of Schreiber *et al.* (51). First, it is possible that c-Fos acts in a tissue-specific and stimulus-specific context to selectively promote or suppress apoptosis. Our observation that c-Fos did not prevent all types of DP thymocyte apoptosis is consistent with this explanation. Second, optimal, but not overexpressed levels of c-Fos, in concert with other factors, could be necessary for cell survival. It will be of interest to investigate apoptosis in *ex vivo* isolated Fos transgenic thymocytes, since this hypothesis would predict that an antisense-mediated down-regulation of c-Fos should protect these thymocytes from apoptosis. Bcl-2 did not appear to require c-Fos for its protective function. However, another family member, Bcl-x, is physiologically highly expressed in DP thymocytes (55), and it will be of interest to determine the dependence of the antiapoptotic function of this protooncogene on c-Fos.

Another observation from our study is that AP-1 (Fos-Jun) presented in the complex with the RelA subunit in activated DP thymocytes (Fig. 3*B*). Consistent with this observation, anti-c-Fos partially suppressed the upper NF-κB complex induced by ionomycin (not shown). The former observation suggested a possible interaction of this complex with AP-1, which was subsequently confirmed using RelA-specific antibodies. As was previously shown for HeLa cells (41), this combinatorial factor is transcriptionally active for the NF-κB-dependent reporter constructs. However, the promoter specificity of the AP-1-RelA complex *in vivo*, and its connection with the regulation of cell survival are unknown at present and are currently under investigation.

Data concerning the AP-1 activity in DP thymocytes are controversial. Very low levels of both AP-1 DNA binding activity and AP-1-dependent transcription were observed after activation of sorted DP thymocytes (56). Chen and Rothenberg (57) also concluded that DP thymocytes are characterized by a strong reduction of AP-1 DNA binding activity. By contrast, Sen *et al.* (58) showed that freshly isolated thymocytes contained high levels of AP-1 activity, which dramatically declined with time following the disruption of the thymocyte-microenvironment contact. Comparison of the two procedures of thymocyte purification used in our study were consistent with the latter finding. DP thymocytes enriched by the rapid panning procedure contained highly inducible AP-1 (Fig. 2), while the ones isolated by a lengthier FCM sorting (not shown) had significantly inferior AP-1 inducibility.

Promoter specificity and regulatory activity of the orphan nuclear receptor NUR-77 are still obscure, but several lines of evidence have implicated this putative transcription factor (or a possible negative regulator of transcription factors) in activation-induced apoptosis of T cell hybridoma (19, 20) and negative intrathymic selection (59). Results presented here are consistent with these findings. All stimuli that resulted in AICD of DP thymocytes induced an early activation of NUR-77 DNA binding activity. It is possible that c-Fos and NUR-77 play opposite roles in death programs of DP thymocytes, the first promoting cell survival and the second being important for cell death control.

Several stimuli investigated in our study strongly activated NF-κB RelA-p50 complex in DP thymocytes. The NF-κB/Rel family of TFs controls transcription of many different genes, including those that play an important role in cell death programs, such as c-*myc* and the genes for p53 and TNFα (60–63). Promoters of the *fas-L* and *fasR* genes also contain putative NF-κB-binding sites (64, 65) although their functional significance is unknown. The mechanism of NF-κB activation is based on the release of RelA-p50 from the cytoplasmic inhibitor IκBα. This release requires at least two modification steps, IκBα phosphorylation and proteolysis, the latter probably being mediated by cysteine proteases or the proteasome (66, 67). Cysteine proteases, especially those from the interleukin-1 converting enzyme (ICE) family, have been implicated as mediators of many types of apoptotic death (reviewed in Ref. 68). Protease inhibitors are known to antagonize apoptosis (69). Thus, our data concerning NF-κB activation, which may be linked to apoptosis, indirectly suggest the possibility that IκBα processing could be an additional target for the antiapoptotic function of protease inhibitors. Experiments are currently in progress to address this possibility.

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