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Regulation of Fas-dependent activation-induced T cell apoptosis by cAMP signaling: a potential role for transcription factor NF-κB

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TCR-mediated activation of T cell hybridomas induces programmed cell death by a Fas-dependent pathway. We now show that costimulation of 2B4 cells, in the absence or presence of transgenic Bcl-2, with anti-CD3ε and forskolin, an activator of cAMP signaling, resulted in antagonism of Fas-dependent activation-induced cell death that was always accompanied by selective down-regulation of the nuclear levels of NF-κB p65-p50 (RelA-p50) transcription factor. Forskolin not only inhibited activation-induced cell death and NF-κB activation, but also suppressed expression of Fas and Fas ligand (Fas-L). Furthermore, NF-κB p65 antisense oligonucleotide down-regulated nuclear levels of NF-κB, inhibited cell surface expression of Fas-L and apoptosis of 2B4. Collectively, these finding demonstrate a potential role of NF-κB in the regulation of activation-induced apoptosis in T lymphocytes.

Keywords: apoptosis; T lymphocyte; NF-κB; Fas ligand

Introduction

Programmed cell death (apoptosis) represents one of the fundamental regulatory mechanisms in development of multicellular organism and in antitumor immunity. During T cell development apoptosis is induced in thymocytes which have failed to express a functional TCR (non-selected or neglected cells) and in thymocytes which express a high-affinity receptor with potential self-reactivity (negative selection). Both types of apoptosis eliminate up to 95–98% of newly generated $CD4^+CD8^+$ thymocytes at any given time (Penninger and Mak, 1994). Activation-induced apoptosis of mature T cells may play a role for maintaining peripheral tolerance and for limiting an antigen-specific T cell response (Ashwell, 1994).

The T cell hybridoma 2B4.11, one of the best models to study cell death, undergoes apoptosis when it is stimulated by its cognate antigen, mAbs to CD3ε or Thy-1, dexamethasone (Dex) (Zacharchuk *et al*., 1990). These stimulators generate distinct intracellular signals that activate specific combination of transcription factors and regulate transcription of genes encoding proteins which ultimately induce or transduce secondary signals leading to cell death (Penninger and Mak, 1994; Krammer *et al*., 1994). The protooncogene Bcl-2 functions to inhibit several types of apoptotic cell death including glucocorticoid-induced apoptosis of T cells. The molecular mechanism by which Bcl-2 inhibits apoptosis is poorly understood although it must affect the nature of the intracellular signals induced by extracellular activating agents (Strasser *et al*., 1991; Nunez and Clarke, 1994).

From many identified transcription factors there is a set of several transcription factor families which are important for a coordinated regulation of gene expression during T cell activation, including NF-κB/Rel, NF-AT, AP-1, Oct, and LEF-1. These transcription factors are targets of TCR-mediated signaling which is dependent from protein kinase C, Ca^{2+} and Ras-Raf pathways (Crabtree and Clipstone, 1994; Chen and Rothenberg, 1994; Baeuerle and Henkel, 1994; Weiss and Littman, 1994). It is likely that some of the same signaling molecules and transcription factors that regulate T cell differentiation and activation are also important regulators of genes controlling commitment to apoptosis.

NF-κB is involved in the transcriptional control of many genes which are expressed in activated lymphocytes, e.g. IL-2, as well as several genes that regulate cell death, such as c-*myc* and tumor suppresser p53 (Baeuerle and Henkel, 1994; La Rosa *et al*., 1994; Wu and Lozano, 1994). NF-κB factors are found in the nucleus as heterodimers of p50 or p52 subunits with three different members of the Rel family: RelA (p65), RelB and c-Rel. RelA, RelB and c-Rel are potent transcription activation subunits while the p50 and p52 subunits have a DNA binding domain but no apparent trans-activator domain. NF-κB p50 or p52 may also form homodimer complexes, which function as negative regulators of NF-κB-dependent transcription *in vivo* (Baeuerle and Henkel, 1994; Baldwin, 1996). NF-κB is present in the cytoplasm as two major precursor forms either a complex RelA-p50 with cytoplasmic inhibitors IκB or a heterodimer RelA-p105. A signal-dependent release of RelA-p50 from its inhibitors is dependent on phosphorylation and subsequent proteolytic degradation of IκB (Thanos and Maniathis, 1995; Baldwin, 1996). Similarly, a second pathway for NF-κB activation is also the result of phosphorylation and proteolytic processing of NF-κB p105-p65 precursor leading to the production and nuclear translocation of RelA-p50 (Palombella *et al*., 1994; Thanos and Maniathis, 1995). The c-Raf kinase was recently found to function in the kinase cascade leading to phosphorylation of IκB and activation of NF-κB resulting in IL-2 production by activated T cells (Li and Sedivy, 1993; Owaki *et al*., 1993). By contrast,

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cAMP signaling and protein kinase A (PKA) have been shown *in vivo* as negative regulators of c-Raf function and NF-κB activation (Chen and Rothenberg, 1994; Cook and McCormick, 1993; Hafner *et al*., 1994; Neumann *et al*., 1995).

Interaction of Fas Ligand (Fas-L) with Fas antigen has been implicated in the generation of a death signaling cascade which is induced by several types of programmed cell death, including activation-induced apoptosis of T cells (Watanabe-Fukunaga *et al*., 1992; Suda *et al*., 1993; Nagata and Golstein, 1995; Dhein *et al*., 1995; Brunner *et al*., 1995; Ju *et al*., 1995; Alderson *et al*., 1995; Yang *et al*., 1995). Forskolin, an activator of cAMP signaling, has been shown to inhibit activation-induced apoptosis (Lee *et al*., 1993) and NF-κB activation (Chen and Rothenberg, 1994, Neumann *et al*., 1995). In present study, we have investigated a potential role of NF-κB in the concert with other transcription factors in the regulation of activation-induced apoptosis of T cells. In addition, we have also evaluated the interrelationship between activation and apoptosis by examining the effects of transgenic Bcl-2 on several transcription factors, including NF-κB, that are normally up-regulated upon T cell activation.

Results

Suppression of activation-induced apoptosis in the 2B4 T cell hybridoma

To evaluate the molecular levels by which TCR-mediated signaling induces cell death and the extent by which Bcl-2 may affect the activation-induced apoptosis, the 2B4 T cell hybridoma was stably transfected with *bcl-2* cDNA in pBMGneo expression vector. 2B4-Bcl-2 cell line with a relatively high level of expression of intracellular Bcl-2 was established. Control 2B4-neo cells were transfected with vector alone (Ivanov *et al*., 1995). Similar to previous studies (Zacharchuk *et al*., 1990; Hockenbery *et al*., 1993), we noted that Dex and anti-CD3 mAb induce apoptosis of 2B4, but overexpression of *bcl-2* in 2B4 only antagonized Dex-induced apoptosis (Figure 1a). Thus, Bcl-2 does not prevent activation-induced apoptosis in these cells. As shown previously (Zacharchuk *et al*., 1990), costimulation of 2B4 cells by Dex and anti-CD3 resulted in antagonism of apoptosis. However, anti-CD3-induced apoptosis of 2B4-neo and 2B4-Bcl-2 was inhibited by forskolin (Figure 1a), an activator of cAMP signaling, that also blocks IL-2 expression (Chen and Rothenberg, 1994). Forskolin alone was poor inducer of death of 2B4 cells (Figure 1a). By contrast, Dex-induced apoptosis was potentiated by cAMP signaling (Jondal *et al*., 1995 and Figure 1a). DNA fragmentation analysis of 2B4-neo (not shown) and 2B4-Bcl-2 cells treated with anti-CD3 and/or forskolin revealed that by 24 h forskolin antagonized anti-CD3-induced apoptosis as evident by the lower fraction of cells containing hypodiploid DNA and the large fraction of cells in the S/G_2-M phases of the cell cycle. Ten μM forskolin alone did not arrest 2B4 cells in G_1 phase (Figure 1b). Forty to fifty mM antioxidant N-acetylcysteine (NAC) also suppressed activation-induced apoptosis of 2B4 cells (Figure 1a).

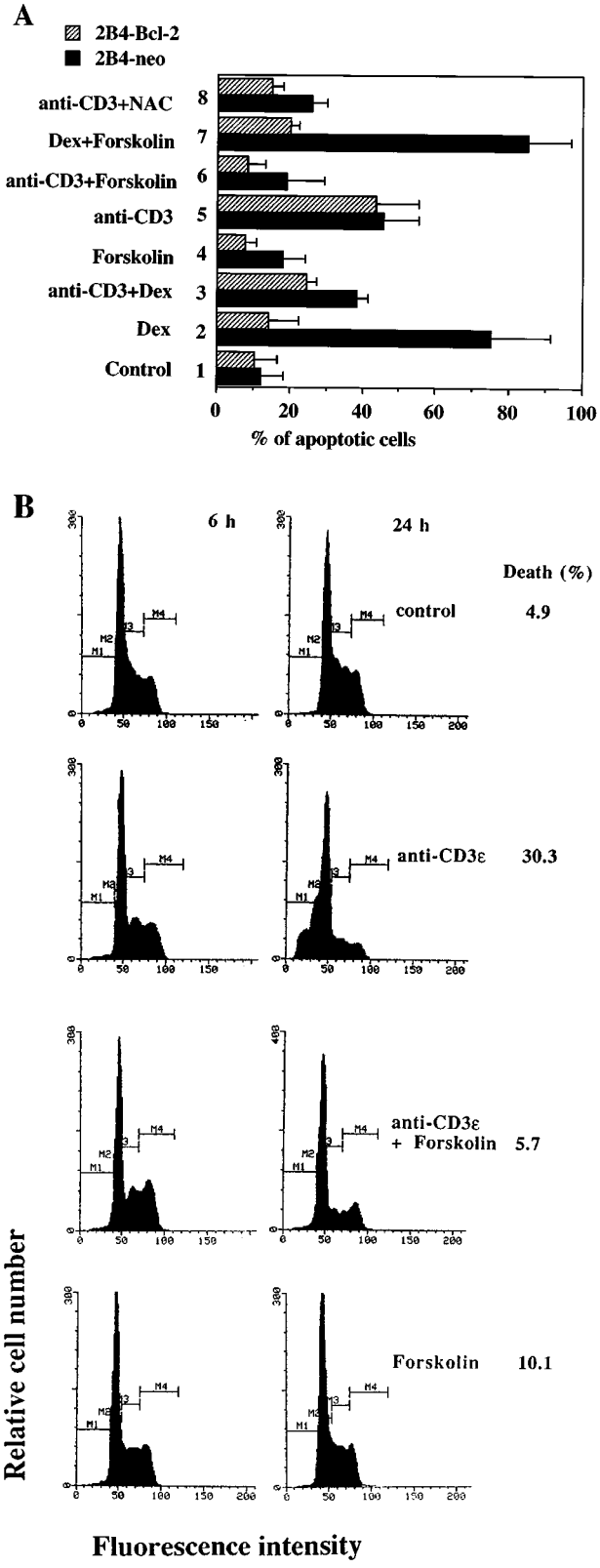


Figure 1 Antagonism of cell death in the 2B4 T cell hybridoma. (**a**) 2B4-neo and 2B4-Bcl-2 (5×10^5 cells/ml) were treated with either 1 μM dexamethasone (Dex), 10 μM forskolin, anti-CD3ε (145-2C11), 50 mM NAC or different combinations of these activators. Percentage of trypan blue positive (dead) cells is shown. (**b**) DNA fragmentation analysis by PI staining and flow cytometry. 2B4-Bcl-2 was cultured with 10 μM forskolin in the presence or absence of anti-CD3ε mAb. The DNA content in the cells was determined 6 h and 24 h after activation. Markers M1, M2, M3 and M4 identify apoptotic cells with hypodiploid DNA content, stage G_1, S and G_2/M of cell cycle, respectively. The percentage of apoptotic cells 24 h after treatment is shown at the right

Activation-induced apoptosis of 2B4 is Fas dependent

Fas-mediated cell death is dependent upon high cell density and engagement of Fas by Fas-L (Brunner *et al*., 1995; Ju *et al*., 1995; Alderson *et al*., 1995; Yang *et al*., 1995). Anti-CD3-induced apoptosis of 2B4-neo (Figure 2a) and 2B4-Bcl-2 was critically dependent upon cell density with highest levels of apoptosis noted at a relatively high concentration of cells ($1-20\times10^5$/ml) in culture. In addition, soluble anti-Fas mAb inhibited anti-CD3-induced apoptosis of 2B4-neo in a dose-dependent manner as assessed by the levels of cells with hypodiploid DNA (Figure 2b, left). As a control, soluble anti-Fas in the absence of anti-CD3 exhibited minimal effect on the cell viability of 2B4-neo, although a somewhat smaller fraction of cells were in G_2-M phase of the cell cycle (Figure 2b, right). These data indicate that the majority of activation induced death of 2B4 is mediated by Fas-dependent pathway.

Forskolin suppresses expression of Fas and the Fas-L

In view of the potent capacity of forskolin to inhibit activation-induced apoptosis of T lymphocytes, we examined whether forskolin might affect the expression of either Fas or Fas-L by 2B4 cells. Northern hybridization analysis revealed that anti-CD3 induced high levels of Fas-L mRNA by both 2B4-neo and 2B4-Bcl-2 6 h after activation, and this induction was almost completely blocked by forskolin (Figure 3a). By contrast, anti-CD3 moderately induced Fas mRNA expression that was most evident after a 24 h culture of 2B4-Bcl-2. When comparing the Fas mRNA levels to the total amount of RNA, as determine by probing for GAPDH mRNA, forskolin appeared to partially inhibit anti-CD3 induced Fas mRNA (not shown).

Cell surface analysis of Fas-L expression on viable cells by FACS analysis revealed that forskolin essentially completely inhibited this protein on 2B4 and 2B4-Bcl-2 cells (Figure 3b, left panel) but only partially blocked expression of surface Fas (Figure 3c). This low levels of Fas-L staining using FITC-Fas-Ig was judged by cold competition by unlabeled Fas-Ig, but not CD30-Ig (not shown). As an additional specificity control, forskolin did not inhibit cell surface expression of other molecules, i.e. Ly6A/E (not shown). Scattergram analysis demonstrated again that forskolin inhibited anti-CD3-induced cell death as assessed by propidium iodide stained cells were detected in the upper portion of each scattergram (Figure 3b, right panel). Collectively, these data indicate that a major target of cAMP-mediated signaling in the process of cell death is the Fas-L and Fas expression.

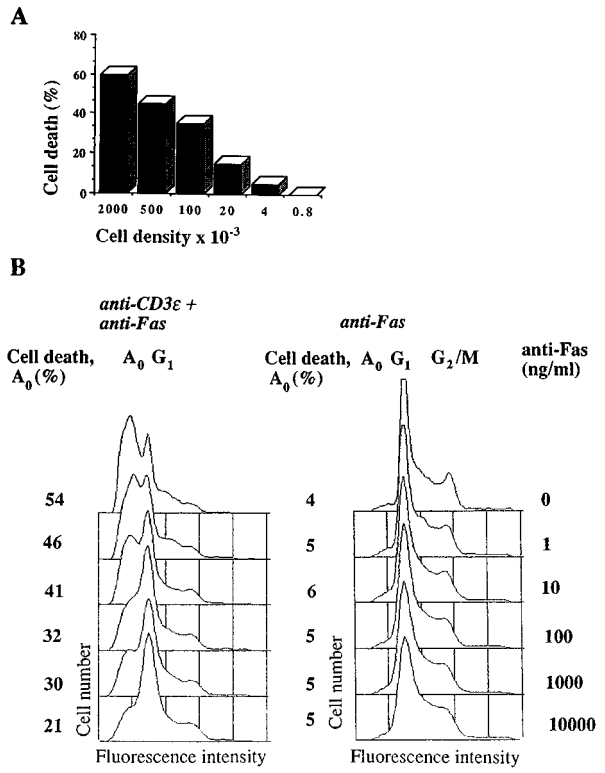


Figure 2 Requirements for Fas/Fas-L for activation-induced apoptosis of 2B4. (**a**) Cell density dependency of cell death. 2B4 cells were activated by immobilized anti-CD3ε at the indicated cell density. The percentage of trypan blue positive (dead) cells was determined 24 h after treatment. (**b**) Effect of soluble anti-Fas mAb on apoptosis. DNA fragmentation analysis was performed in the presence of the indicated concentration of soluble anti-Fas and immobilized anti-CD3ε. DNA content was determined by PI staining and flow cytometry. Percentage of apoptotic cells (A_0) is shown on the left

Positive and negative regulation of transcription factors in activated 2B4 cells by forskolin

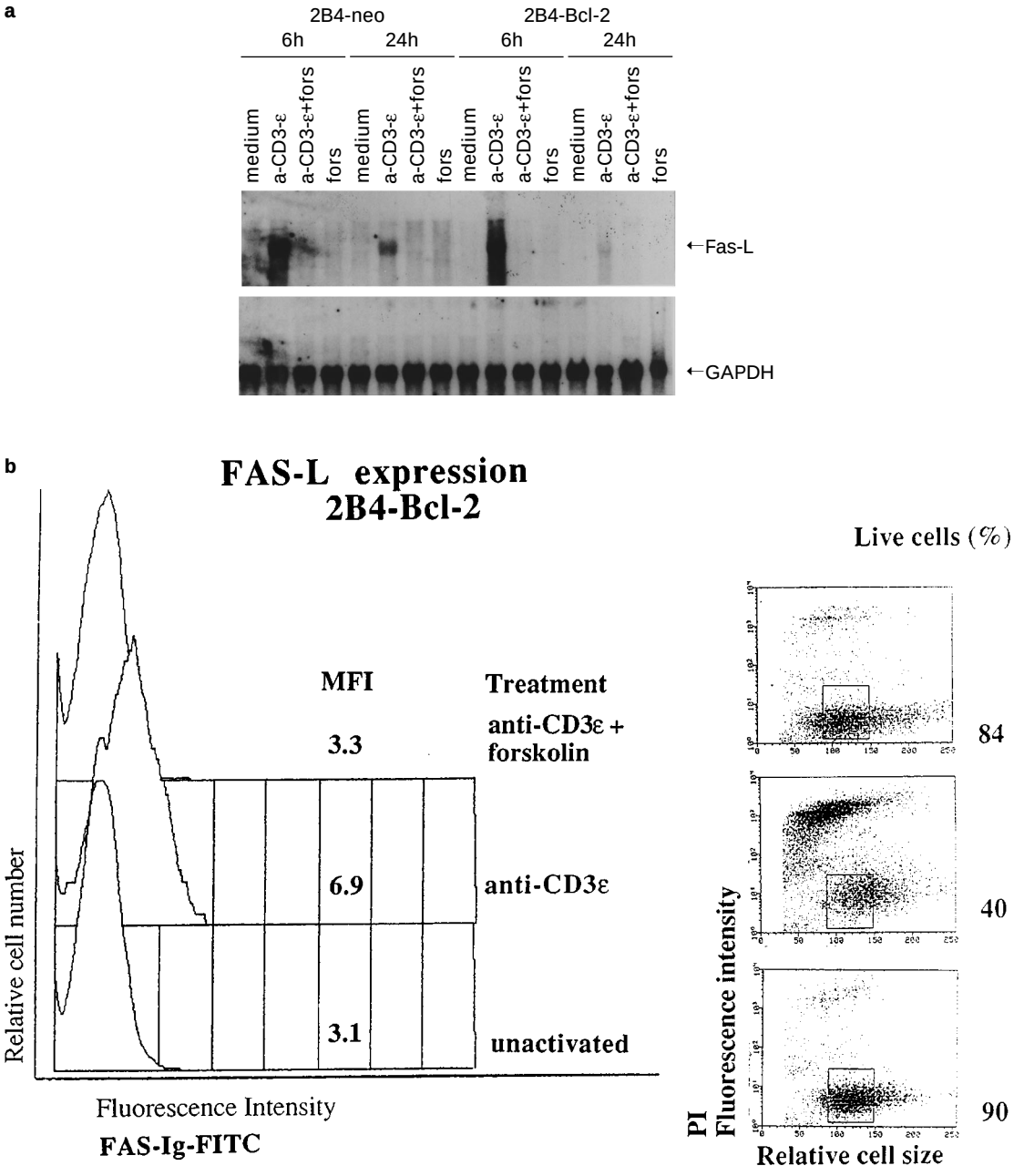
To determine whether transcription factor activities are selectively regulated by forskolin costimulation, we examined binding activities of several transcription factors in the beginning (6 h of stimulation with anti-CD3) and at the final phase of apoptotic commitment of cells (24 h of stimulation) in the presence or absence of forskolin (Figure 4a and not shown). As expected, EMSA experiments showed induction or up-regulation of several transcription factor activities 6 h after anti-CD3 treatment, including NF-κB p65-p50 (RelA-p50) and RelB-p50, which comigrate as the upper band of NF-κB DNA binding complexes, AP-1 and LEF-1 (Figure 4a). As expected, the CREB/ATF family, a known target of cAMP signaling (Bridle and Montminy, 1992), was directly regulated by forskolin. The intensity of band b2, which probably represents an unphosphorylated CREB factor, was substantially decreased whereas bands b3 and b4 (probably, phosphorylated forms) were strongly up-regulated in presence of forskolin (Figure 4a). Forskolin and anti-CD3 costimulation up-regulated the levels of AP-1, and this may be dye increased heterodimerization of c-Jun with CREB/ATF by cAMP signaling (Hai and Curran, 1991). By contrast, forskolin negatively affected the basal and mAb-induced levels of binding activity of LEF-1 in both 2B4-neo and 2B4-Bcl-2. For Oct-1, a slight positive effect of forskolin was detected for 2B4-neo, but a negative effect for 2B4-Bcl-2 (Figure 4a). These data established that cAMP signaling by forskolin exerted distinct effects on the nuclear levels of several transcription factors that are up-regulated upon T cell activation by anti-CD3.

The strongest inhibitory effect by forskolin and anti-CD3 costimulation was observed for NF-κB p65-p50

(Figure 4a). By identification of NF-κB complexes from 2B4 cells in EMSA (Ivanov *et al*., 1995) based on inhibition and supershifts after pretreatment of nuclear extracts with specific antibodies to p65 and RelB, very little NF-κB p65-p50 complex was detected after forskolin and anti-CD3 costimulation whereas RelB-p50 was still easily detected. The lower band of NF-κB complexes represented p50-p50 homodimer. A quite similar pattern of transcription factor levels was also observed 24 h after treatment when the cells were undergoing maximal levels of activation-induced apoptosis (not shown). These results correlate enhanced levels of NF-κB p65-p50 trans-activator for T cells undergoing cell death. By contrast, cAMP signaling led to a significant reduction of cell death and was accompanied by down-regulation of p65-p50 binding activity. As was previously described (Ivanov *et al*., 1995), both untreated and activated 2B4 cells contained very low levels of NF-AT activity. Costimulation of 2B4 in presence of forskolin down-regulated these levels (not shown).

NAC and Dex affect transcription factor binding activities and activation-induced apoptosis 2B4 cells

We examined whether NF-κB p65-p50 was also down-regulated in other situations in which anti-CD3-induced apoptosis was suppressed. Costimulation 2B4 cells with anti-CD3 mAb and 50 mM NAC resulted in suppression of apoptosis (Figure 1a) and down-regulation of nuclear levels of NF-κB p65-p50 and LEF-1, while AP-1 activity was not substantially affected (Figure 4b). Twenty-five mM NAC did not effectively decrease these levels. In addition, Dex antagonism of anti-CD3-induced stimulation also resulted in reduced levels of p65-p50 whereas RelB-p50 and p50-p50 were minimally affected. Costimulation by anti-CD3 and Dex also resulted in decreased



levels of AP-1 and LEF-1 (Figure 4c). In recent publications (Yang *et al*., 1995; Williams and Henkart, 1996), down-regulation of Fas-L levels after costimulation of T cells by anti-CD3 in the presence of Dex or NAC was also observed. Hence, down-regulation of p65-p50 was directly correlated with suppression of activation-induced apoptosis for all three cases of the costimulation 2B4 cells in the presence of apoptotic inhibitors, while changes in the levels of AP-1 and LEF-1 might be positive or slightly negative.

Functional role for NF-κB p65-p50 in the regulation of Fas-L and apoptosis of 2B4 cells

Although the molecular experiments correlated decreased levels of NF-κB p65-p50 in each case when anti-CD3-induced apoptosis was also inhibited, the data do not demonstrate a direct functional role for p65-p50 in activation-induced apoptosis. Therefore, the capacity of sense and antisense NF-κB p65 oligonucleotides on anti-CD3-induced apoptosis of 2B4-Bcl-2 was tested. As an additional non-specific control RelB sense oligonucleotide was used. Experiments with labeled oligonucleotides showed a very similar uptake of all three oligonucleotides into cells (not shown). At a dose 1–10 μg/ml these oligonucleotides were not toxic and did not affect cell growth. Partial inhibition of 2B4 cell growth was detected at higher concentration of antisense and sense oligonucleotides (20 μg/ml, not shown). At a dose of 1 μg/ml (Figure 5b) and 5 μg/ml (Figure 5a), the antisense p65 oligonucleotide inhibited the capacity of anti-CD3 to induce cell surface expression of Fas-L (left panel) and cell death (right

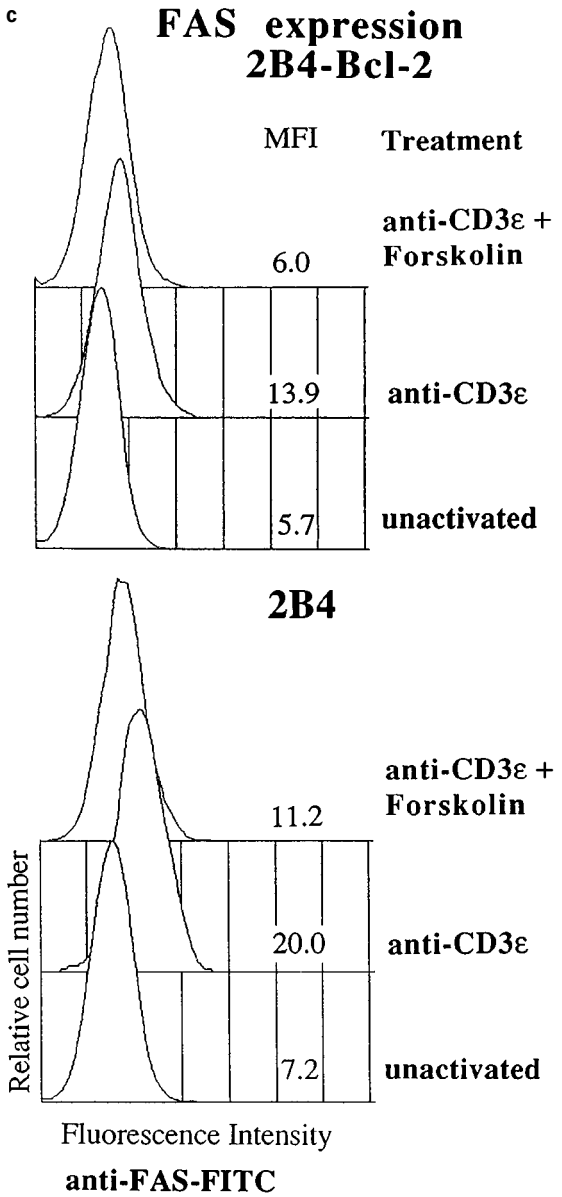


Figure 3 Expression of Fas and Fas-L by activated T cell hybridoma. (**a**) Northern hybridization analysis. 2B4-neo and 2B4-Bcl-2 were cultured as indicated in the Figure in the presence or absence of forskolin (fors). Total RNA was isolated 6 and 24 h after culture initiation and probed for Fas, Fas-L and GAPDH mRNA. (**b**) Cell surface expression of Fas-L. 2B4-Bcl-2 were activated as indicated for 24 h and stained with Fas-Ig-FITC and PI and analysed by flow cytometry. Percentage of live cells, which was detected by PI exclusion, is shown at the right. Only live cells, i.e. cells found in the box of each scattergram (right of Figure), were analysed for Fas-L expression to generate the histograms at the left. MFI represents the medium fluorescence intensity. (**c**) Cell surface expression of Fas. 2B4 and 2B4-Bcl-2 were stained by anti-Fas-FITC mAb

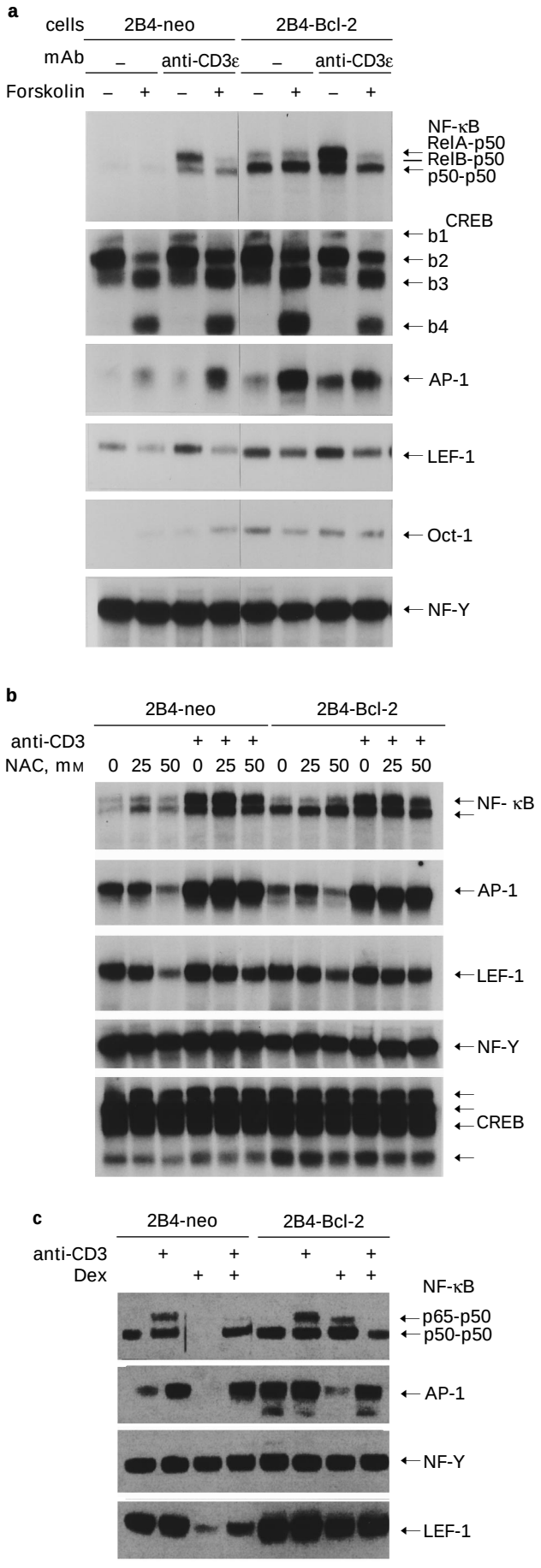


Figure 4 Effect of forskolin (**a**), NAC (**b**) and Dex (**c**) on transcription factor levels in 2B4. 2B4-neo and 2B4-Bcl-2 were activated as indicated. EMSAs were performed with nuclear proteins 6 h after 2B4 cells activation with ^{32}P-labeled oligonucleotide probes. Specific DNA-binding complexes are shown by arrows

panel). These concentrations of oligonucleotides had no effect on the cell surface expression of Ly-6A/E (not shown). An irrelevant oligonucleotide (sense RelB) did not substantially inhibit anti-CD3-induced apoptosis or Fas-L expression (not shown). Control EMSA experiments (Figure 5c) and Western blot analysis (Figure 5d) also demonstrated that the NF-κB p65 antisense oligonucleotide specifically inhibited the up-regulation of the nuclear levels p65-p50 and p50-p50 after anti-CD3 treatment, while effect of sense oligonucleotide was minimal. Others have also observed that antisense p65 oligonucleotide inhibited levels NF-κB p50-p50 (Kitajima *et al*., 1992). This effect may be due to secondary inhibition of p50 transcription, which is under control of NF-κB (Baeuerle and Henkel, 1994), by down-regulation of p65-p50 levels.

Discussion

Our data indicate a potential role for NF-κB in the regulation of T cell apoptosis. This factor appears to be involved in activation of one or more genes necessary for Fas-dependent cell death. This conclusion is supported by following observations. (i) Activation of NF-κB (RelA-p50), Fas/Fas-L expression and activation-induced apoptosis are inhibited by cAMP signaling. Furthermore, in the two additional cases of inhibition of activation-induced T apoptosis with costimulation in the presence of NAC or Dex, a down-regulation of NF-κB p65-p50 (present study) and Fas-L levels (Yang *et al*., 1995; Williams and Henkart, 1996) was observed. (ii) A NF-κB antisense p65 oligonucleotide inhibited nuclear levels of NF-κB, Fas-L expression, and death of 2B4 cells. (iii) The putative NF-κB element of Fas-L promoter region GGAAACTTCC (Takahashi *et al*., 1994) that differs from the canonical κB motif (GGGRNNYYCC) only by one nucleotide, may bind NF-κB p65 subunits in the concert with other factors (not shown). However, we have not received data concerning a crucial role of this site for transcription of Fas-L gene. An additional study of the transcriptional control of the Fas-L gene expression is necessary.

We have used the 2B4 T cell hybridoma as a model cell system. This cell line and its variants have been widely used to study TCR-mediated activation of IL-2 production and subsequent cell death (Zacharchuk *et al*., 1990; Codias *et al*., 1990; Ivanov *et al*., 1994). Three agents, i.e. forskolin, NAC and Dex, antagonized anti-CD3-induced apoptosis. Evaluation of the nuclear levels of six distinct transcription factors that are often induced upon TCR-mediated signaling showed that NF-κB p65-p50 was the only transcription factors that was substantially down-regulated in all cases of costimulation of 2B4. The nuclear level of LEF-1 was also usually lower, but in this case the effect was often modest. Thus, these data correlated decrease of NF-κB p65-p50 with

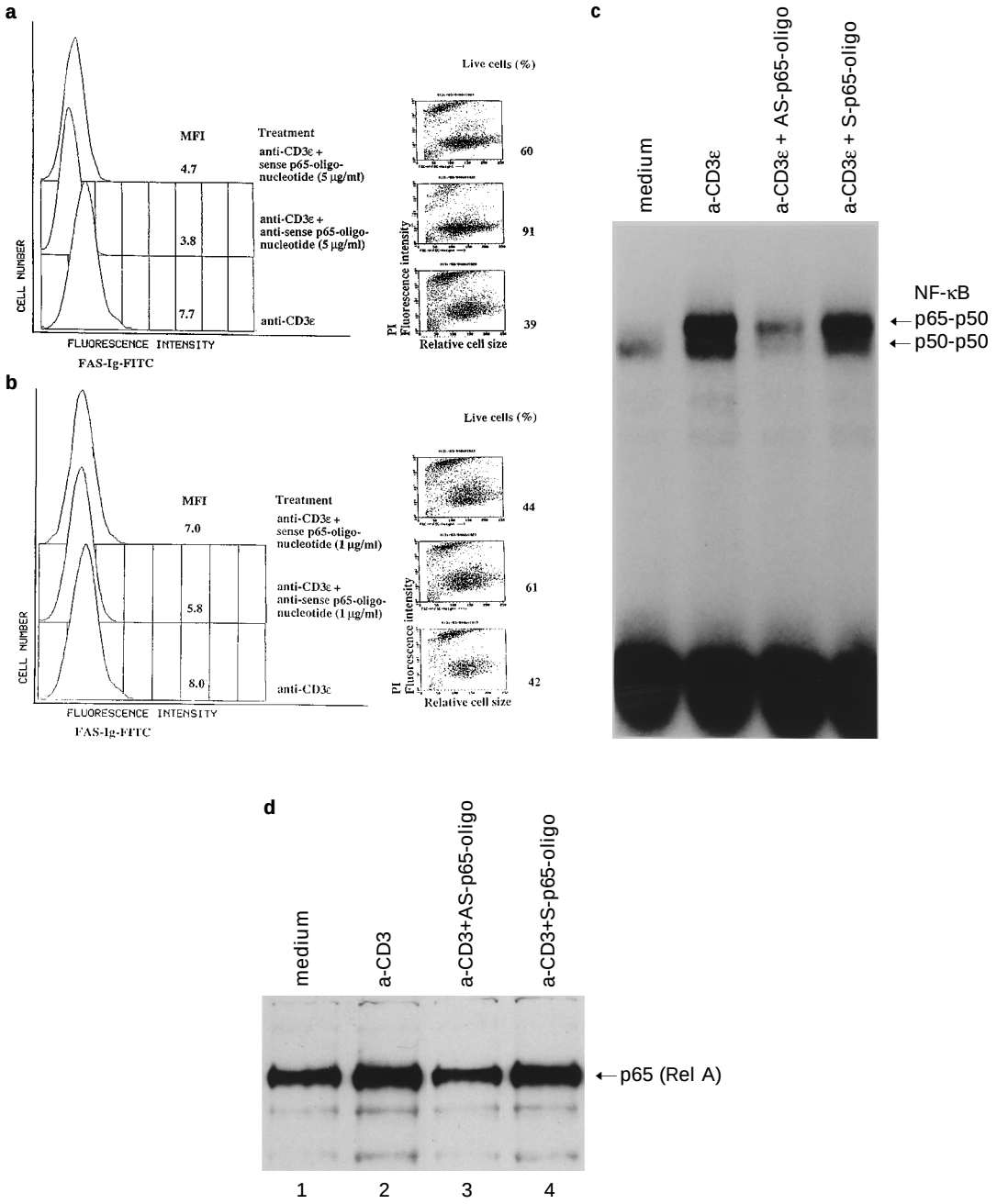


Figure 5 Effect of NF-κB p65 antisense oligonucleotide on Fas-L expression and apoptosis of 2B4 cells. 2B4-Bcl-2 were activated with immobilized anti-CD3 mAb, as indicated, for 24 h in the presence of 5 μg/ml (**a**) or 1 μg/ml (**b**) of the indicated oligonucleotide. Cells were stained by Fas-Ig-FITC (left side) and PI and analysed by flow cytometry. The cells in the lower portion of the scattergram at the right represent viable cells. These cells were analysed for Fas-L expression. NF-κB p65-50 DNA binding activity (**c**) and p65 protein levels (**d**) of oligonucleotide treated cells. EMSA (**c**) and Western blotting (**d**) were performed with nuclear extracts of 2B4-Bcl-2 that were treated as for 24 h as listed above each lane with antisense (AS) or sense (S) NF-κB p65 oligonucleotides

inhibition of apoptosis. Importantly, the presence of transgenic Bcl-2 in 2B4 cells exerted a minimal effect on antagonizing activation-induced apoptosis, and similar molecular changes were noted for 2B4-neo and 2B4-Bcl-2 cells.

There has been some controversy regarding the role of Bcl-2 in protecting T cells from activation-induced apoptosis. Similarly to the observation of others (Green *et al*., 1994), overexpression of Bcl-2 by 2B4 did not protect theses cells from anti-CD3-induced cell death. By contrast, this level of Bcl-2 very efficiently protected 2B4 from Dex-induced apoptosis (Hockenbery *et al*., 1993). The reason for this difference is currently not known. Overexpression of Bcl-2 by 2B4 resulted in higher preactivated nuclear levels of several transcription factors, including NF-κB p65-p50, p50-p50, AP-1 and NF-AT. Since stimulation by anti-CD3 up-regulates transcription factors, the net effect maybe potentiation of apoptosis rather than inhibition of cell death even in the presence of Bcl-2. Alternatively, Bcl-2 may exert its anti-apoptotic activity by selectively acting on glucocorticoid receptor mediated signaling and transcription factor activity induced in 2B4 by Dex (Ivanov *et al*., 1995).

Similar to results by others (Nagata and Golstein, 1995; Dhein *et al*., 1995; Brunner *et al*., 1995; Ju *et al*., 1995; Alderson *et al*., 1995; Yang *et al*., 1995), engagement of the TCR by anti-CD3 resulted in substantial induction of Fas-L expression relatively early after activation. Fas mRNA and cell surface protein levels were also induced. Thus, the molecular determinants necessary for Fas-dependent apoptosis were expressed by anti-CD3 activated 2B4. We demonstrated that the majority of anti-CD3-induced apoptosis by 2B4 is dependent upon Fas/Fas-L pathway as judged by the relatively high cell density required for cell death and by capacity of anti-Fas mAb to inhibit the majority of the cell death. We cannot rule out that the portion of cell that was resistant to anti-Fas mAb may occurred by a Fas-independent pathway.

Past work has demonstrated activation-induced cell death is inhibited by forskolin (Lee *et al*., 1993) and prostaglandin E2 (Goetzl *et al*., 1995) suggesting that this biological process was down-regulated upon cAMP signaling. The molecular basis accounting for this phenomenon was unknown. Our data indicated that forskolin inhibited Fas-dependent apoptosis by down-regulation of cell surface levels of Fas-L and to lesser extent Fas. Another well characterized target of cAMP signaling is NF-κB. Forskolin has been shown to act as a potent suppressor of activation of NF-κB p65-p50 (Chen and Rothenberg, 1994; Neumann, 1995). Our observation of blockage of apoptosis, Fas-L expression and NF-κB p65-p50 activation by cAMP signaling and antisense p65 oligonucleotide has causally associated NF-κB with induction of Fas-dependent apoptosis. Additionally, NF-AT activity is also suppressed by cAMP-mediated signaling. Although we have not established the key target gene(s) which are regulated by NF-κB and/or NF-AT for Fas-dependent cell death, one attractive candidate is the expression of the Fas-L gene itself. Analysis of the 5′ regulatory region of the Fas-L gene has revealed a relatively restricted set of binding sites for known transcription factors, including NF-κB (Takahashi *et al*., 1994). Our analysis of the capacity of this element to bind to NF-κB demonstrated relatively weak binding of RelA subunit when compared to NF-κB binding to HIV promoter (not shown). Furthermore, slight but detectable levels of NF-ATp binding by the same Fas-L site was also observed in preliminary experiments. These data suggest that combinatorial binding both NF-κB and NF-AT subunits to this region may be functionally relevant. Possible interactions between NF-κB and NF-AT transcription factor families (both families belong to the Rel superfamily) in the regulation of transcription were previously described (McCaffrey *et al*., 1992; Casolaro *et al*., 1995).

Two other transcription factors have been implicated in the regulation of activation-induced apoptosis, c-Myc (Shi *et al*., 1992) and orphan receptor Nur-77 (Liu *et al*., 1994; Woronicz *et al*., 1994). A precise role for Nur-77 in transcriptional regulation has not yet established. However, NF-κB has been shown to control transcription of c-myc (La Rosa *et al*., 1994), indicating a possible interrelationship between these two factors in the regulation of apoptosis. It is also interesting to note that TNF receptor associated protein TRADD not only signals cell death but also activates NF-κB (Hsu *et al*., 1995). In this case the signals leading to cell death and NF-κB activation were shown to be distinct. By contrast to present study, for the TNF-dependent systems activation of NF-κB prevented or down-regulated apoptosis (Wang *et al*., 1996; Beg and Baltimore, 1996; Liu *et al*., 1996; Van Antwerp *et al*., 1996). These very interesting data, as well as our former data concerning a role of NF-κB for T cell development (Ivanov *et al*., 1993), demonstrated a dual (positive or negative) role of NF-κB in the apoptotic regulation of different cell systems. Although results of the present study have implicated NF-κB in Fas-mediated apoptosis, it does not preclude a broader role for this transcription factor in regulation of programmed cell death. In this respect, very promising observation was recently reported concerning phenotype of IκBα knockout mice (Beg *et al*., 1995). These mice are characterized by constitutive NF-κB (RelA-p50) activation, thymic atrophy and reduction of proportion of DP thymocytes 6 days after birth that indicated, probably, a role of RelA-p50 in thymocyte apoptosis. In other hand, recently described NF-ATp knockout mice (Hodge *et al*., 1996) were characterized by very strong reduction of induced Fas-L levels in T cells that further indicated a role of NF-AT for the regulation of Fas-L expression.

Materials and methods

Cells

The 2B4 T cell hybridoma (Zacharchuk *et al*., 1990) and its transfected variants were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 50 μM 2-mercaptoethanol and antibiotics. 2B4 cells were transfected with *bcl-2* cDNA in the pBMGneo expression vector (Karasuyama and Melchers, 1988; Deng and Podack, 1993) by electroporation and selected for G-418 resistance. A cell line with high-level expression of *bcl-2* mRNA was established on the basis of Northern blot analysis (2B4-Bcl-2). Control cells were 2B4 transfected with the unmodified vector pBMGneo (2B4-neo) (Ivanov *et al*., 1995)

Antibodies

The mAb 145-2C11 to CD3ε (Leo *et al*., 1987) has been described previously. The mAb to mouse Fas (Jo2) and the polyclonal Ab to mouse RelA (p65) were purchased from PharMingen (San Diego, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

Fas-Ig construction

Mouse Fas-Ig cDNA was constructed by fusing mouse Fas cDNA, truncated at the extracellular domain next to the transmembrane segment (fragment A) with the γ chain of mouse IgG1 (fragment B) starting at the hinge region. These cDNAs were synthesized by RT–PCR, simultaneously introducing XhoI cloning sites and mutating the three cysteines of the IgG1 hinge to serines. For construction of fragment A, RNA was isolated from mouse thymocytes, transcribed into cDNA, and PCR was performed using the primers to produce the extracellular domain of murine Fas with 5′ XhoI end and 3′ mouse IgG1 linker (primer 1–5′-ATC CCT GGG CAC GCG ATT TCT GGG ACT TTG TTT CCT-3′ and primer 2–5′-TTT CTC GAG ATT TTC ACT CCA GAC ATT GTC CTT C-3′). Fragment B was amplified by RT–PCR using RNA

isolated from a mIgG1 secreted hybridoma (primer 3 – 5′-CCC AGA AAT CGC GTG CCC AGG TCT GGT TCT AAG-3′ and primer 4 – 5′-AGC TTT CTC GAG AAG GAC ACT GGG ATC ATT TAC CAG G-3′) so fragment B contained a 5′ ends complementary to the 3′ ends of the fragment A and had 3′ XhoI ends. Fragments A and B were fused by PCR using primers 1 and 4 to produce Fas-Ig cDNA which was cloned into plasmid PCR II (Invitrogen, San Diego, CA).

The Fas-Ig cDNA was subcloned into XhoI site of pBMGneo (Karasuyama and Melchers, 1988) and transfected into NIH3T3 cells. Fas-Ig fused protein was purified from supernatants of transfected cells using the Protein A MAPS System ('Bio-Rad', Hercules, CA). The Fas-Ig was conjugated with FITC Isomer I on Celite (Calbiochem, La Jolla, CA).

T cell activation and flow cytometry

2B4 cells were activated with anti-CD3ε in 24-well flat-bottom plates that were coated with purified anti-CD3 (10 μg/ml) for 3 h prior to initiation of culture. Forskolin (10 μM), NAC (20 – 50 mM) Dex (1 μM) (Sigma, St Louis, MO) were added as indicated.

2B4 was directly stained with an excess of FITC-Fas, FITC-anti-Ly-6A/E, FITC-Fas-Ig, FITC-anti-CD3. Cells were pretreated with 2.4G2 mAb to block FcR. Flow cytometric analysis was performed on FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Antisense oligonucleotide treatment

The sense and antisense phosphorothioate analogs of the oligonucleotides to the 5′ end of NF-κB p65 mRNA, including the ATG initiation codon, were synthesized [NF-κB p65-sense oligonucleotide CTG ACC ATG GAC GAT CTG TTT CCC, NF-κB p65-antisense oligonucleotide GGG AAA CAG ATC GTC CAT GGT CAG (Nolan *et al*., 1992; Kitajima *et al*., 1992)]. As non-specific control, RelB sense oligonucleotide was used [CGG GCC ATG AAG AGT CGC CGC GCT (Ryseck *et al*., 1992)]. Oligonucleotides were used at the final concentration in the range of 1 – 5 μg/ml of culture media. Cells were first preincubated for 4 h with oligonucleotides following by stimulation with anti-CD3 for additional 16 h.

DNA fragmentation analysis

After fixation with 70% ethanol in PBS, cells were pelleted and resuspended in 0.5 ml of PBS containing propidium iodide (40 μg/ml) and DNase-free RNase A (10 mg/ml). Cells were incubated at 37°C for 30 min and then analysed on a FACScan (Becton Dickinson, CA).

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):

agctTGGGGACTTTCCAGCCG for NF-κB; agctTGATGAGTCAGCCG for AP-1; agctCCATGACGTCATGG for CREB; AAAGAGGAAAATTTGTTTCATACAGAA for NF-AT; GAAAATATGTGTAATATGTAAAACATTTTG for OCT; AAAAAAGAACACAAAGGGCCTAG-ATT for LEF-1; GTCTGAAACATTTTTCTGATTGGTTAAAAGTTGAGTGCT for NF-Y.

These oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Nuclear extracts were prepared from $5-15\times10^6$ T cells. Binding reactions were carried out by incubating the end-labeled DNA (50 000 c.p.m.) with 5 μg of nuclear proteins and 2 μg of poly(dI-dC) as previously described (Ivanov *et al*., 1993).

Northern hybridization analysis

Cytoplasmic cell extracts were used for isolation and purification of total RNA by SDS-phenol method. Total RNA was separated on formaldehyde/MOPS agarose gel and blotted on Hybond N membranes (Amersham, Arlington Heights, IL) which were hybridized with ^{32}P-labeled DNA probes for Fas (Watanabe-Fukunaga *et al*., 1992), Fas-L (Suda *et al*., 1993), and GAPDH.

Western blot analysis

Nuclear extracts were resolved on a 10% SDS – PAGE, transferred to nitrocellulose and processed by standard manual. The polyclonal Ab against RelA wasa used at dilution 1 : 1000. The secondary Ab was goat anti-rabbit IgG conjugated to horseradish peroxidase (dilution 1 : 4000). Signals were detected using the ECL system (Amersham, Arlington Heights, IL).

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