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Biochemical and kinetic characterization of the glucocorticoid-induced apoptosis of immature $CD4^+CD8^+$ thymocytes

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Abstract

We characterized kinetic and biochemical changes during glucocorticoid (GC)-induced apoptosis of immature $CD8^+CD4^+$ double-positive (DP) thymocytes. A GC analog dexamethasone (Dex) induced rapid apoptotic commitment and a transient up-regulation of the NF-κB/RelA-p50-binding activity in DP cells. This required an early activation of proteasome, as judged by the ability of a specific proteasomal inhibitor, lactacystine, to delay apoptosis and to suppress Dex-dependent NF-κB activation. Dex-induced apoptotic commitment was preceded by the rapid (3 h) cleavage of both a typical caspase substrate, poly(ADP-ribose) polymerase (PARP), and of nuclear transcription factors AP-1, NF-κB p50–p50 and NUR-77. By contrast, phorbol myristate acetate (PMA) and/or ionomycin-induced apoptosis had much slower kinetics, were preceded by an early increase of NF-κB/RelA-p50, AP-1 and NUR-77 activities, and were insensitive to proteasome inhibition. Both the transgenic Bcl-2 and zVAD-fmk, an inhibitor of caspases, affected all features of Dex-induced apoptosis in a similar fashion, by inhibiting cell death and PARP cleavage, and by stabilizing AP-1, NF-κB p50–p50 and NUR-77 levels. Furthermore, Bcl-2 prevented Dex-induced RelA-p50 activation. However, a higher gene dosage of the transgenic Bcl-2 was required for protection against Dex, compared to the PMA and/or ionomycin-induced apoptosis. These findings highlight the unique mechanistic features of GC-induced apoptosis.

Introduction

The vast majority of developing thymocytes (95–98%) die by apoptosis. The eliminated cells die by two fundamentally distinct mechanisms. The first category of cells fail positive selection (non-selected or neglected cells), and are therefore believed to die by true 'programmed' death. Indeed, $CD4^+CD8^+$ double-positive (DP) thymocytes have a strictly limited lifespan of ~3 days in the absence of positive selection (1). The second group of cells die because they bear potentially autoreactive receptors (negative selection) (2). This type of thymocyte death is induced by extracellular signals that operate via the TCR and is reminiscent of the activation-induced cell death (AICD) of mature peripheral T cells exposed to antigen. The mechanism of death by neglect is still unknown; while some evidence points to the role of endogenous glucocorticoid (GC) (3–5), the involvement of other signaling pathways cannot be excluded at present. Evidence also exists for the mutually antagonistic effect of GC and TCR stimulation in determining life and death during selection (6). The precise mechanism of death during negative selection is also incompletely understood, but is likely to involve, in addition to the TCR signaling, a secondary death signaling cascade, that may use one or more members of the tumor necrosis factor (TNF)/nerve growth factor (NGF) ligand–receptor family pairs (7,8). Critical interactions in the secondary cascade, however, remain obscure.

The goal of this study was to investigate biochemical and kinetic characteristics of the GC-induced thymocyte death, as a possible model of programmed death of non-selected thymocytes. Cell death and the induction of transcription factor (TF) DNA-binding activities were investigated following GC stimulation in the presence or the absence of apoptotic and/or proteolytic inhibitors Bcl-2 (9), zVAD-fmk [a caspase inhibitor (10–12)] and lactacystin (a proteasome inhibitor), to evaluate the role of various proteolytic mechanisms in this type of thymocyte apoptosis, and to compare it to other types of apoptosis, chiefly the ones induced by pharmacologic

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agonists of TCR signaling. As in our previous study (13), we focused on inducible TF of the NF-κB/Rel family (14,15), AP-1 (16), NUR-77 (17) and CREB/ATF (18), because these TF control and coordinate expression of numerous genes during T cell development, differentiation and programmed death. NF-Y, a TF with stable DNA-binding activity (19), was used as a reference factor. Several unique biochemical and kinetic features were observed, most notably at the level of proteasomal involvement and TF regulation. These features were not shared by phorbol ester and/or ionophore-induced apoptosis, indicating that these types of apoptosis are divergent in the upstream pathways and that they probably converge relatively late, near or at the point of caspase activation.

Methods

Mice

Female C57BL/6 (B6 mice) (NCI Animal Facility, Frederick, MD) and *bcl-2* transgenic mice (9) (from Dr S. Cory, WEHI, Melbourne, Australia via Dr H. T. Petrie, MSKCC, New York, NY) were used at 4–8 weeks of age. *bcl-2/+* mice used in this study were back-crossed for at least 10 generations to C57BL/6 and were typed for the *bcl-2* transgene by the PCR, as described (9). *bcl-2/bcl-2* homozygous mice were obtained from the mating of *bcl-2/+* × *bcl-2/+* mice.

Thymocyte preparation, activation and flow cytometry

DP thymocytes were enriched from total thymocytes by 'panning' with an anti-CD8 mAb (3.155), immobilized on the surface of plastic dishes as described previously (20). CD4$^+$CD8$^+$ thymocytes were treated with immobilized anti-CD3ε mAb (145-2C11; PharMingen, San Diego, CA). Ionomycin (250 ng/ml), phorbol myristate acetate (PMA; 10 ng/ml), dexamethasone (1 μM), LLnL (10–50 μM) (all from Sigma, St Louis, MO) and the caspase inhibitor, zVAD-fmk (10–50 μM) (Enzyme Systems, Dublin, CA), were added as indicated. Lactacystin, the proteasome inhibitor, was purchased from Dr E. J. Corey (Harvard University, Cambridge, MA) and used at 10 μM. Annexin-V–FITC/propidium iodide (PI) staining to detect early apoptotic cells was performed using TACS Annexin-V–FITC kit (Trevigen, Gaithersburg, MD). Flow cytometric analysis was performed on a FACScan flow cytometer using Lysys II software (Becton Dickinson, Mountain View, CA) by analyzing 5×10^3 cells/sample using wide light scatter gates to include late apoptotic cells.

DNA fragmentation analysis

This assay was performed as described previously (21). Cells were pelleted and resuspended in 0.5 ml of hypotonic buffer with 0.1% Triton X-100 containing PI (40 μg/ml) and DNase-free RNase A (10 mg/ml). Cells were incubated at 37°C for 30 min and then analyzed on a FACScan (Becton Dickinson). 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. Analysis was performed without light scatter gating.

Oligonucleotides and electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotides used as specific probes for transcription factors and the EMSA were described previously (22). DNA binding activities of TF were quantified using the BioRad Molecular Imaging system (model G5-250), equipped with Molecular Analysis software (BioRad, Hercules, CA). Intensities of bands are given in the text and in the figure legends.

Western blotting analysis

Nuclear and cytoplasmic extracts were resolved on a 10% SDS–PAGE and transferred to nitrocellulose. The polyclonal anti-poly(ADP-ribose) polymerase (PARP) serum (Biomol, Plymouth Meeting, PA) was used at dilution 1:1000. The polyclonal anti-NF-κB p50 antibodies (to NLS peptide; Santa Cruz Biotechnology, Santa Cruz, CA; or to the whole p50 protein, a gift of Dr M. Lenardo, NIH), polyclonal antibody against RelA and c-Fos (Santa Cruz Biotechnology), as well as mAb against NUR-77 (a gift of Dr B. Osborne, University of Massachusetts, Amherst, MA) were used at a dilution of 1:1000. The secondary antibodies were goat anti-rabbit IgG or goat anti-mouse Ig conjugated to horseradish peroxidase (dilution 1:4000). Signals were detected using the ECL system (Amersham, Arlington Heights, IL). Transgenic human Bcl-2 was detected using a hamster mAb (clone 6C8; PharMingen) followed by treatment with goat anti-hamster IgG labeled with biotin and streptavidin–horseradish peroxidase, as previously described (23).

Results

Dex-induced apoptotic commitment of DP thymocytes

DP thymocytes from B6 mice, enriched to the final purity of 91 ± 2%, were treated for up to 18 h with Dex, PMA or ionomycin. For all reagents used in this study, extensive dose titrations were performed (not shown) and the doses exhibiting maximal apoptosis induction used throughout the study. Apoptosis was evaluated by detecting phosphatidylserine residues exposed at the surface of apoptotic cells (annexin-V–FITC staining in the presence of PI) (24) and/or by measuring DNA fragmentation in single nuclei of permeabilized cells as the percentage of nuclei to the left of the diploid $G_{0/1}$ peak, diagnostic of hypodiploid cells that have lost DNA (21). Dex induced high levels of DP thymocyte apoptosis as early as 3–6 h after treatment, as detected by annexin-V–FITC staining (not shown and Fig. 1A). This early apoptosis was effectively suppressed in the presence of cycloheximide, indicating a requirement of new protein synthesis. Determination of the kinetics of apoptotic commitment, measured by the extent of DNA loss, after stimulation via different signaling pathways indicated that Dex-induced apoptosis had much more rapid kinetics than apoptosis induced via the TCR signaling agonists PMA or ionomycin (Figs 1B and 3B).

Since Dex-induced death is known to require new gene expression (25) (Fig. 1A), we evaluated TF regulation during the apoptotic commitment of DP thymocytes. Special attention was devoted to AP-1 and NF-κB families of TF, which appear to be the master regulators of numerous genes during T cell activation and under stress conditions. DNA-binding activities of TF in the nuclear extracts of stimulated DP cells were assessed using the EMSA. Identification of nuclear TF from DP thymocytes in EMSA experiments using non-labeled oli-

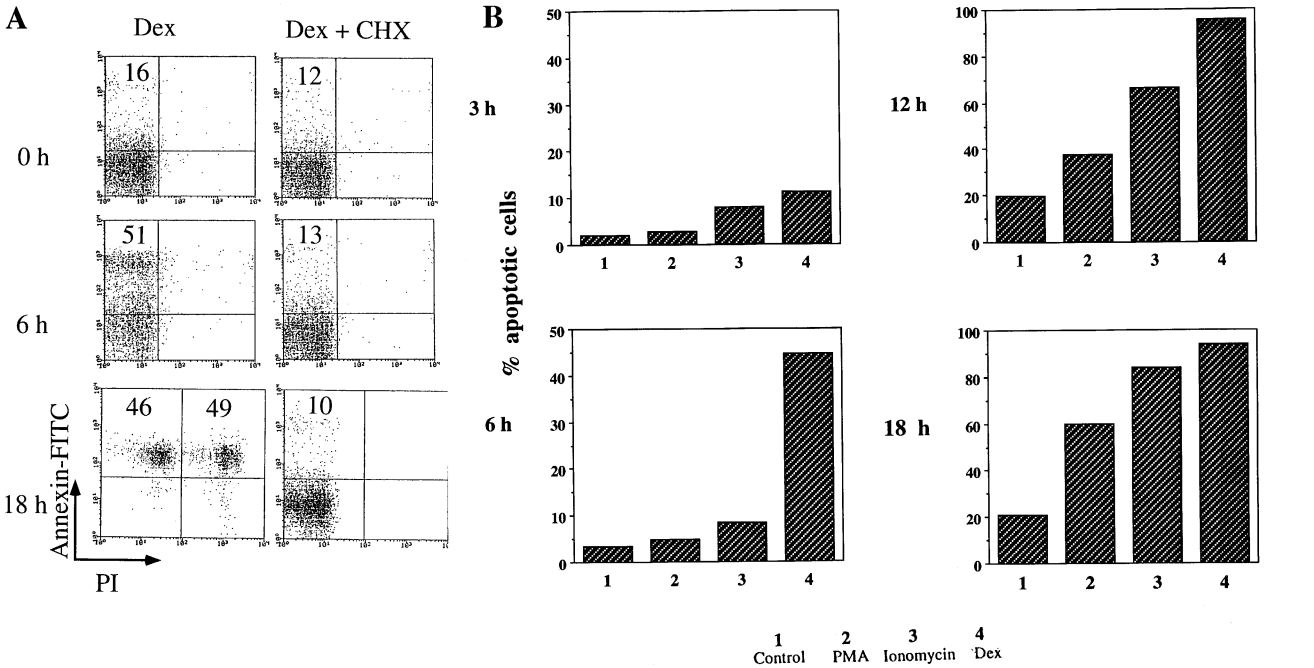

Fig. 1. GC receptor-mediated apoptosis of DP thymocytes. DP thymocytes from normal B6 mice were stimulated for 3–18 h by Dex (1 μM), ionomycin (250 ng/ml) and PMA (10 ng/ml). Percentage of early apoptotic cells (A) was determined by Annexin-V–FITC and PI staining. Results are representative of five experiments. The percentage of cells undergoing DNA fragmentation (B) was determined as described in Methods and (21), at indicated time points. Results are representative of 15 experiments.

gonucleotide competition and specific antibody pretreatment was reported previously (13). In all experiments, as in our previous study (13), the presence of basal TF binding activity was always observed, owing to constitutive *in vivo* stimulation of DP thymocytes, many of which are undergoing positive or negative selection, as well as differentiation and terminal proliferation. Thus, all of the observed changes are judged against that background. Dex induced a moderate up-regulation of RelA-p50 (the upper NF-κB band) between 3 and 6 h after treatment (Fig. 2A, lanes 3 and 4). Of note, the kinetics of the RelA-p50 up-regulation after Dex treatment was quite different from that induced by the TCR agonist PMA or a cytokine TNF-α, the two typical activators of the Rel family of TF. In the two latter cases, nuclear translocation of RelA-p50, as a result of IκBα degradation, was evident 30 min after treatment (not shown), while in the case of Dex stimulation the IκBα levels increased at 1 h due to positive regulation of the IκBα transcription by Dex (27,28) with subsequent down-regulation 3 h after treatment (not shown) and a consequent nuclear translocation of RelA-p50 (Fig. 2A). This NF-κB RelA-p50 up-regulation appeared to be a by-product of proteasomal activation and consequent IκBα degradation (15) (see also Fig. 4C). More importantly, Dex-induced signaling also resulted in a remarkable and quick down-regulation of basal levels of other TF, including NF-κB p50–p50, AP-1 (the upper band = Jun–Fos complex; the lower band = Jun–Jun complex) and NUR-77 (Fig. 2A, lanes 1–4). Decrease in the DNA binding activity was clearly manifested at 3 h (Fig. 2A), preceding the DNA fragmentation which was first detectable at 6 h (Fig. 1B). Down-regulation reached 60% for NF-κB p50–p50, 80% for AP-1 Jun–Fos, 60% for Jun–Jun, 95% for the upper NUR-77 complex and 40% for CREB 6 h after treatment (Fig. 2A). By contrast, control cells incubated without stimulation (Fig. 2A) or cells stimulated with PMA (Fig. 2B) or ionomycin (not shown) did not exhibit any TF degradation over the same time period.

Since caspase-dependent proteolysis is involved in many types of apoptosis (reviewed in 10,11), we performed Western blot analysis of nuclear proteins from Dex-treated thymocytes to detect cleavage of PARP, a typical target protein of the caspase cascade and an indicator of early apoptotic commitment (28). PARP cleavage produces an 85 kDa fragment from an intact 116 kDa molecule. Such a change was detectable 3 h after Dex treatment with a subsequent increase in the level of the cleaved 85 kDa fragment 6 h after treatment (Fig. 2C). As normal DP thymocytes undergo significant spontaneous apoptosis, it was not surprising that the intact p116 PARP was detected in these cells at reduced levels, and that DP thymocytes incubated with Dex and the caspase inhibitor zVAD-fmk (see Fig. 3D) exhibited higher levels of intact p116 PARP than control cells. Furthermore, Dex-induced PARP cleavage was substantially suppressed in *bcl-2* transgenic cells (not shown). Proteolysis of the NF-κB p50 subunit occurred in parallel to that of PARP, as detected by the use of antibodies to both the whole p50 (Fig. 2C) and to the nuclear localization sequence peptide (not shown), indicating a cleavage of the 4 kDa fragment from the N-end of p50. (The resulting polypeptide was designed as p50Δ.) A pattern of partial proteolytic degradation (minor bands among the main NUR-77 species) after Dex treatment was also observed

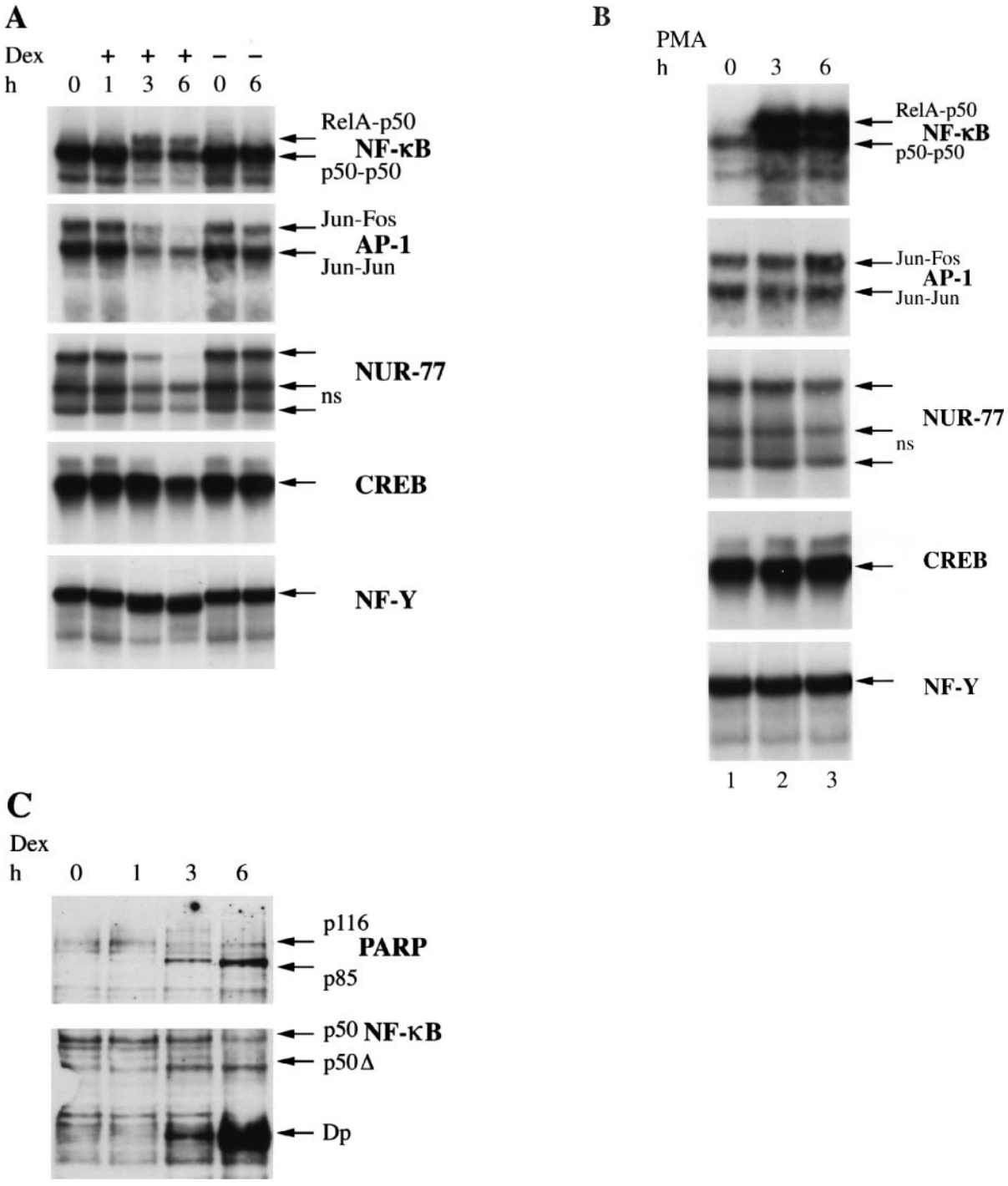

Fig. 2. Dex-induced changes in nuclear TF levels of DP thymocytes. (A and B) EMSA were performed with nuclear proteins isolated 0–6 h after control, 1 μM Dex (A) or 10 ng PMA (B) treatment of thymocytes. Arrows indicate the position of specific DNA-binding complexes of NF-κB, AP-1, NUR-77, CREB and NF-Y; ns, non-specific band. Free labeled oligonucleotide probes are not shown. (C) Nuclear protein levels of NF-κB p50 and PARP in Dex-treated thymocytes. Western blot analysis was performed using polyclonal antibodies to NF-κB p50, and PARP, as described in Methods. Dp, degraded protein.

for NUR-77 (not shown). Thus, the caspase cleavage of its typical substrate, PARP, and proteolysis of NF-κB p50 and NUR-77 proceeded with parallel kinetics, suggesting that these TF may be among the caspase substrates. We conclude that Dex-induced reduction in nuclear TF binding activities is a consequence of proteolytic degradation of TF. Of interest, such cleavage was not observed in any of the experiments with other apoptosis inducers (PMA and/or ionomycin) within the first 12 h of stimulation, at which time DNA fragmentation became clearly detectable (e.g. Fig. 1B, 12 h).

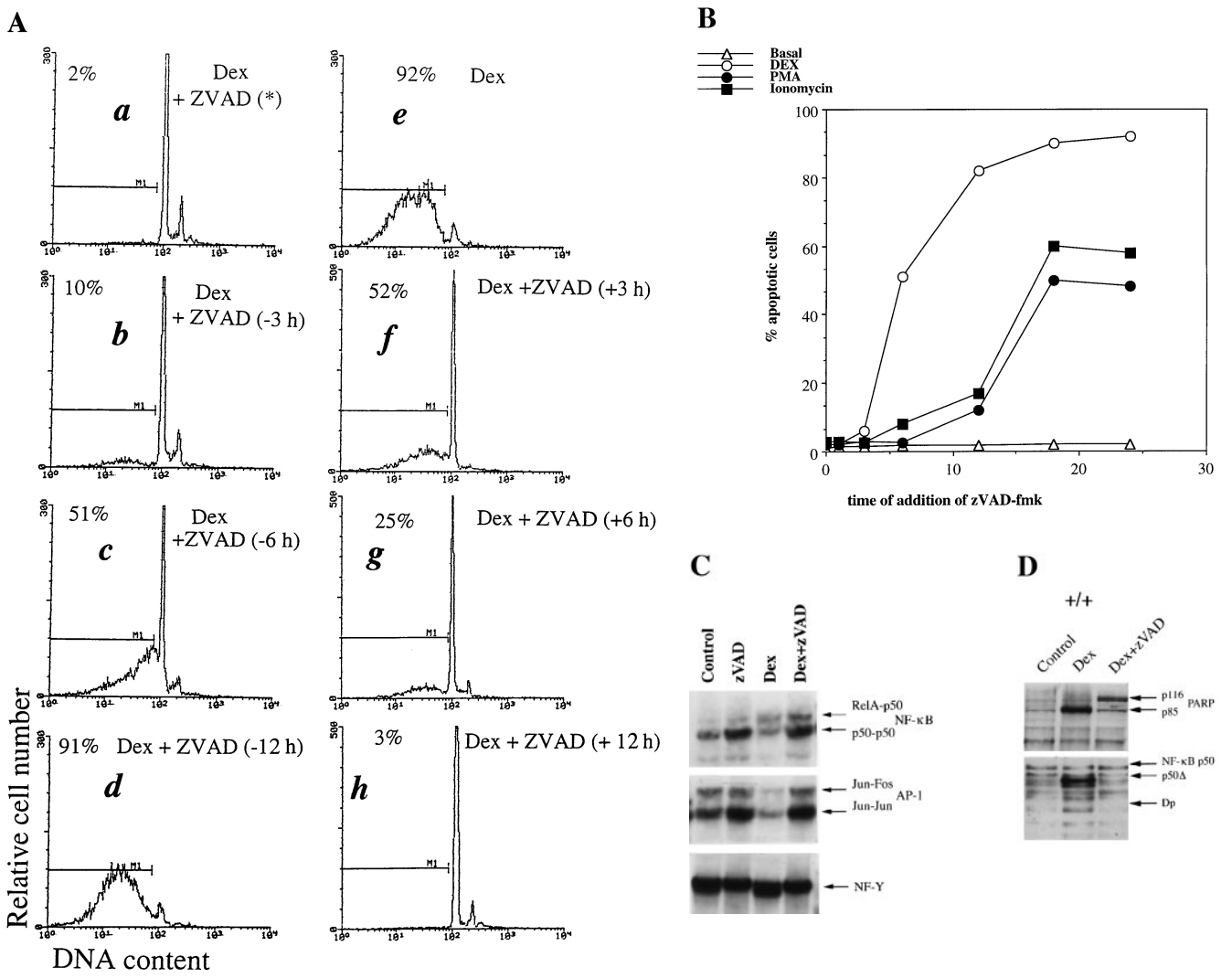

Fig. 3. Kinetics of caspase inhibition and of the reversal of Dex-induced thymocyte apoptosis. (A and B) Reversibility of Dex-induced apoptosis. (A) DP thymocytes were treated simultaneously with Dex + zVAD-fmk (50 μM) (a) or with Dex alone (e), and were left with these reagents for 18 h. Alternatively, cells were incubated with Dex from time 0 and zVAD-fmk added at the indicated time delays indicated by negative numbers (b–d); or were incubated with Dex + zVAD-fmk for the indicated time period, thoroughly washed and re-plated in normal medium (f–h). The percentage of cells undergoing DNA fragmentation was determined after 18 h of total culture. (B) Cells were treated with ionomycin (250 ng/ml), PMA (10 ng/ml) or 1 μM Dex for 24 h and zVAD-fmk, that was added 0, 1.5, 3, 6, 12 and 18 h after culture initiation. The percentage of cells undergoing DNA fragmentation was determined by PI analysis. (C) Effect of zVAD-fmk on Dex-regulated levels of transcription factors. EMSA were performed with nuclear extracts of normal DP thymocytes treated for 6 h with 1 μM Dex in the presence or absence of 50 μM zVAD-fmk, as indicated above the lanes. (D) Nuclear levels of NF-κB p50 and PARP were determined by Western blotting. DP thymocytes were treated by Dex for 3 h in the presence or absence of z-VAD-fmk.

Inhibition of the caspase family of proteases blocks Dex-induced thymocyte apoptosis and the degradation of TF

Given a critical and, possibly, universal involvement of caspases in apoptosis (10-12), we elected to further investigate the role of proteolysis in Dex-induced thymocyte apoptosis, using an irreversible peptide inhibitor of the caspase family of proteases, zVAD-fmk. A 50 μM concentration of the inhibitor was not toxic and by itself did not induce cell death. zVAD-fmk, added at the onset of Dex stimulation, effectively suppressed Dex-induced thymocyte apoptosis up to 18 h after treatment (Fig. 3A and B) (29). We next examined the kinetics of caspase activation. For the Dex-induced apoptosis, a 3–6 h delay in the introduction of zVAD-fmk substantially increased cell death levels, demonstrating a crucial role of rapid caspase activation in this type of apoptosis (Fig. 3A). Consistent with this idea, incubation of thymocytes with Dex + zVAD-fmk for 6 h with subsequent rewashing and transfer of cells into the fresh media without Dex and zVAD-fmk was accompanied by a significant (~70%) protection against apoptosis, compared to cells treated for 6 h by Dex alone (Fig. 3A, *cf.* panels g and e, and B). A 6 h delay in the introduction of zVAD-fmk to Dex-stimulated thymocyte cultures also resulted in DNA fragmentation in a substantial percentage (>50%) of cells (Fig. 3A, panel c, and B). By contrast, the same delay in the

introduction of zVAD-fmk had no effect on PMA- or ionomycin-induced apoptosis (Fig. 3B), suggesting that mechanistic differences exist between Dex- and PMA/ionomycin-induced apoptosis. However, zVAD-fmk also suppressed the late stage of PMA/ionomycin- (Fig. 3B) or anti-CD3-induced (not shown) apoptosis, indicating that common final steps of the cell execution in both cases must involve caspase activation.

We next investigated whether cleavage of nuclear TF may also be blocked by inhibiting caspases. In the case of Dex-induced apoptosis, down-regulation of both the AP-1 complexes and NF-κB p50–p50 (Fig. 3C) and NUR-77 (not shown) 6 h after treatment was completely suppressed by the caspase inhibitor zVAD-fmk, as detected by EMSA; increased stability of nuclear RelA-p50 (in concert with increased NF-κB p50–p50 levels) was also observed, suggesting that the induction of RelA-p50 complex by Dex is not, by itself, the determining factor of apoptotic commitment (Fig. 3C). Western blot analysis confirmed suppression of PARP cleavage, NF-κB p50 degradation (Fig. 3D) and NUR-77 degradation (not shown) in the presence of zVAD-fmk at the protein level. These results establish that caspase activation is essential for the degradation of nuclear TF, and demonstrate that stabilization of nuclear protein levels of TF NF-κB p50–p50, AP-1 and NUR-77 by zVAD-fmk correlates very well with the suppression of apoptotic commitment of DP thymocytes after Dex stimulation.

Involvement of the proteasome in early apoptotic commitment after Dex treatment of DP thymocytes

RelA-p50 nuclear translocation results from the ubiquitin-proteasome-dependent degradation of its phosphorylated cytoplasmic inhibitor, IκBα (15). The observed up-regulation of the RelA-p50 levels in the nuclear fraction 3 h after Dex treatment (Fig. 2A), which was similar to the one observed after anti-CD3 stimulation of thymocytes (13), therefore suggested the involvement of the proteasome in mediating GC receptor (GR) and TCR signaling. To test this hypothesis, we evaluated the effects of lactacystin, a highly specific inhibitor of the proteasome (30), on thymocyte apoptosis induced by Dex or by TCR agonists. By itself, lactacystin did not affect spontaneous thymocyte apoptosis levels 6 h after treatment (Fig. 4A), although it did stabilize the basal pre-existing level of RelA-p50 activity (up-regulation of 1.2- to 1.5-fold compared to the basal level in untreated cells; Fig. 4C). Lactacystin (10 μM) provided partial protection to Dex-treated DP cells 6 h after treatment (Fig. 4A) and led to a down-regulation of the Dex-induced nuclear RelA-p50 activity (2.5-fold increase; Fig. 4C, lane 3) to basal levels (Fig. 4C, lane 4) and stabilization of NF-κB p50–p50 levels. Furthermore, the decrease of AP-1 activity induced by Dex was also suppressed in the presence of lactacystine, consistent with the fact that both Jun and c-Fos protein components of AP-1 represent the targets of the ubiquitin-proteasome pathway (31,32). Levels of CREB and NF-Y activities were stable (<5% variation).

Effects of proteasome inhibition on Dex-induced death were, however, transient. At 12 h after Dex treatment in the presence of lactacystin, no suppression of Dex-induced apoptosis was evident. At this time point lactacystin alone induced substantial levels of apoptosis (Fig. 4A). This was accompanied by a drastic degradation of TF, characteristic for the advanced phase of apoptosis (not shown). Addition of fresh lactacystine at 4, 8 and 12 h of culture did not change the kinetics or the outcome of apoptotic commitment, indicating that the observed phenomena were not simply due to the short half-life of the inhibitor. Very similar results were obtained using 25 μM LLnL (calpain inhibitor I), a peptide inhibitor of the proteasome with a somewhat broader specificity than lactacystin (not shown). The implications of these findings are addressed in the Discussion.

While lactacystin suppressed the RelA-p50 activation induced by αCD3 mAb or by PMA, ionomycin or PMA ionomycin (not shown), this suppression and proteasome inhibition had little or no effect on apoptosis induced by these reagents (not shown and Fig. 4B), even when lactacystin was added at multiple times during culture (not shown). These results demonstrated that inhibition of the proteasome resulted in the transient delay of Dex-induced thymocyte apoptosis, suggesting the existence of both proteasome-dependent and proteasome-independent branches of the GR-mediated apoptotic commitment. Inhibition of both the proteasome (at early time points) and the caspase cascade had very similar effects on stabilization of TF activities, suggesting the possibility of a cross-talk or sequential activation between these two proteolytic systems. By contrast, apoptosis induced by the TCR agonists tested was entirely independent of the proteasome activity. Finally, prolonged inhibition of the proteasome was by itself an apoptotic stimulus, consistent with the recently reported results (33), but inhibition of caspases also suppressed this type of apoptosis (not shown).

Effects of transgenic Bcl-2 on Dex-induced apoptosis of DP thymocytes

Transgenic Bcl-2 effectively protected against PMA- and ionomycin-induced apoptosis of DP thymocytes, but suppressed Dex-induced apoptosis only in the first 6 h (not shown, and Fig. 5B and C). At the same time, transgenic Bcl-2 modulated changes in TF and caspase activity induced by Dex. RelA-p50 activity was not induced, while the down-regulation of the Jun–Fos and the upper NUR-77 complexes was slower after 3 h, compared to non-transgenic DP cells. In parallel, at the same time point, PARP and NF-κB cleavage was suppressed (not shown).

To investigate whether this partial effectiveness can be overcome by increasing the gene dosage of *bcl-2*, we took advantage of *bcl-2* homozygous transgenic thymocytes, containing double copy number of the *bcl-2* and the correspondingly increased protein levels of Bcl-2 as detected by Western blotting (Fig. 5A). For these thymocytes, Dex-induced death was effectively suppressed, in a manner indistinguishable from that of the caspase inhibition 12 h after treatment (Fig. 5C). However, even these high levels of Bcl-2 still had virtually no effects on the anti-CD3-induced cell death (not shown). As expected, down-regulation of AP-1 binding activity (both Jun–Fos and Jun–Jun complexes) 6 h after Dex treatment was substantially suppressed in *bcl-2/bcl-2* transgenic thymocytes (Fig. 5C). At later times, *bcl-2/bcl-2* DP thymocytes were still partially protected (viability of 40–50% compared to unstimulated controls, as compared to 5–10% viability of *bcl-2/* or non-transgenic DP cells). This partial protection correlated with detectable levels of TF binding activities as

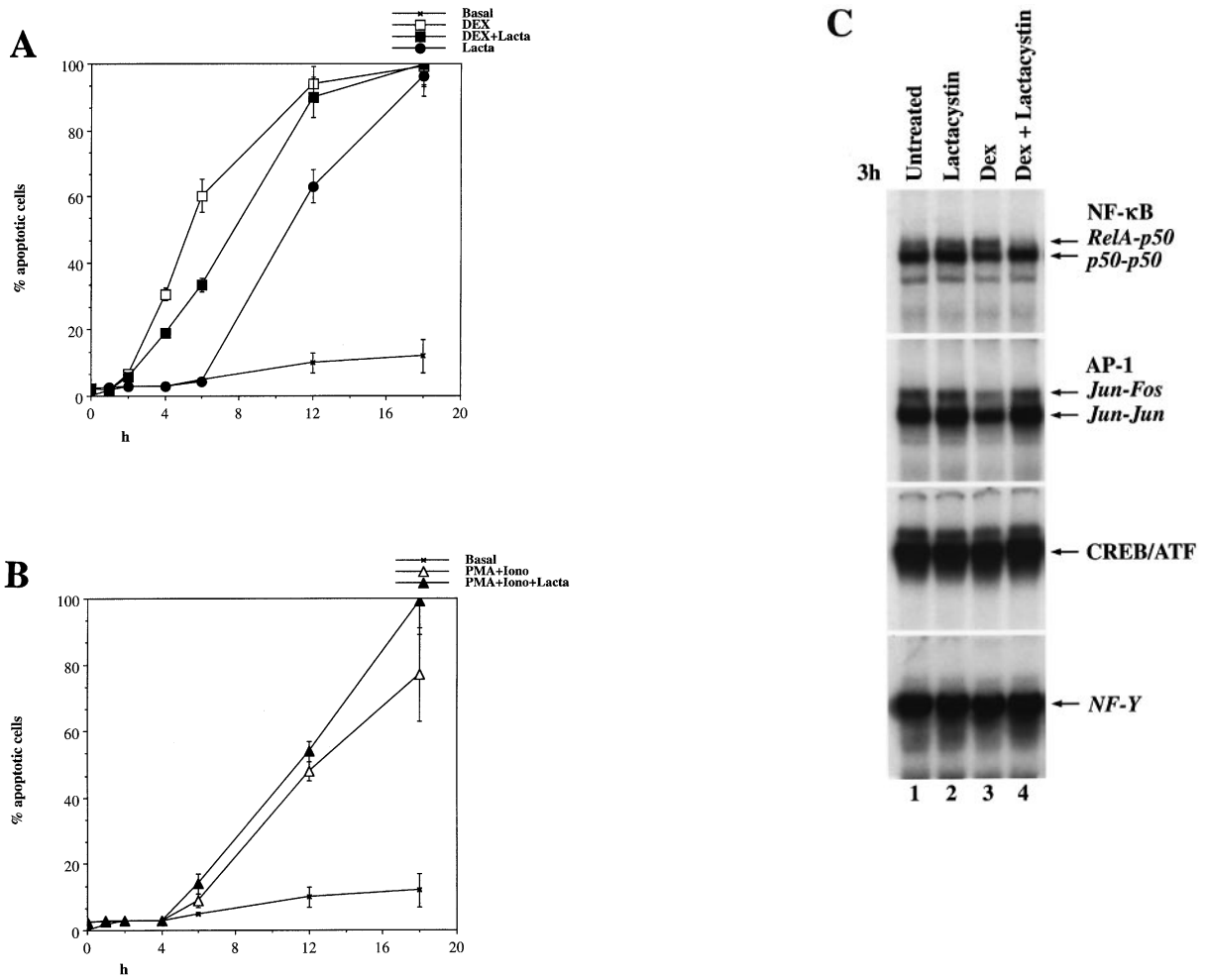

Fig. 4. Effect of lactacystin on Dex- or PMA ionomycin-induced apoptosis of DP thymocytes. DP thymocytes were treated with Dex (1 μM) (A) or ionomycin (250 ng/ml) plus PMA (10 ng/ml) (B) in the presence or absence of 10 μM lactacystin (Lacta) or were left untreated. Apoptosis (%) was measured by Annexin-V–FITC and PI staining thymocytes 0–18 h after treatment. Fresh lactacystine was also introduced into the culture at 4, 8 and 12 h of culture. (C) EMSA were performed with nuclear proteins isolated 3 h after 1 μM Dex treatment of thymocytes in the absence or the presence of 10 μM lactacystin, as indicated above the lanes. Arrows indicate the position of specific DNA-binding complexes of NF-κB, AP-1, CREB and NF-Y. Free labeled oligonucleotide probes are not shown. Results are representative of three experiments.

late as 24 h after Dex treatment of Bcl-2/Bcl-2 transgenic DP thymocytes (Fig. 5C), albeit this activity was much weaker than the one observed at 6 h. Degradation of NF-κB p50–p50 and NUR-77 was also suppressed (not shown). This finding further corroborates the notion that the TF integrity and activity tightly correlate with the viability of cells during GC-induced DP apoptosis.

Discussion

It has been proposed that death of non-selected thymocytes during thymocyte development may be dependent on endogenous GC (3–5) while negative selection of thymocytes is, in good part, reminiscent of AICD of mature T cells (34–37). Evidence from another study suggested that these two pathways may antagonize each other *in vivo* (6). Our results suggest that GC-induced apoptosis proceeds largely via signaling cascades different from the ones invoked by other apoptosis inducers, converging with them only distally, near or at the point of caspase activation. The unique characteristics of the Dex-induced apoptosis of DP cells observed in the present study relate to the kinetics of apoptotic commitment, the TF regulation, the proteasome involvement and the protective effects of Bcl-2. Dex-induced apoptotic commitment in DP thymocytes was very rapid, and was characterized by a relatively late RelA-p50 nuclear translocation and an early caspase-dependent degradation of nuclear proteins, including PARP and TF AP-1, NUR-77 and NF-κB p50–p50. Dex-mediated induction of RelA-p50 (caused by IκB degradation) was suppressed and the apoptosis delayed by the inhibition of the proteasome, as well as by the presence of the transgenic Bcl-2. In all cases, Dex-induced apoptosis correlated strictly to the proteolytic degradation of nuclear TF activity. By contrast, PMA and/or ionomycin-induced

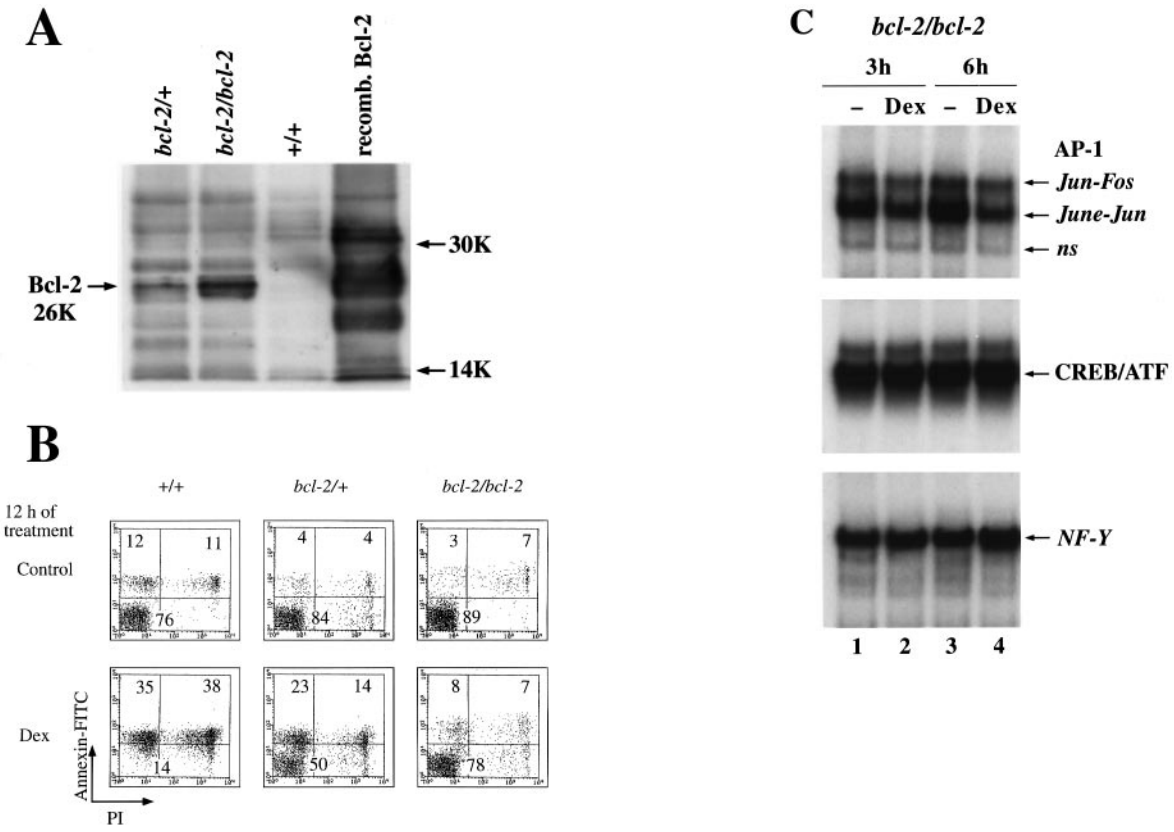

Fig. 5. Gene dosage effect of the transgenic *bcl-2* on Dex-induced apoptosis. (A) Western blot analysis of Bcl-2 expression in DP thymocytes from heterozygous *bcl-2/ ,* homozygous *bcl-2/bcl-2* and normal, non-transgenic mice. (B) Apoptosis in DP thymocytes of mice shown in (A), following 12 h of treatment with 1 μM Dex, and detection with Annexin-V–FITC and PI. (C) EMSA of nuclear proteins of DP thymocytes of *bcl-2/bcl-2* following Dex treatment. Arrows indicate the positions of AP-1, CREB and NF-Y complexes.

apoptosis (this paper) as well as that induced by TCR cross-linking (not shown) was much slower, and was accompanied by an initial up-regulation of nuclear TF activity. This TF regulation did not correlate to the outcome of the PMA- or ionomycin-induced apoptosis. Furthermore, this type of death was characterized by a late caspase activation (12 h), was independent of the proteasome and, at least in the case of the relevant TCR cross-linker, the anti-CD3 mAb, could not be inhibited by transgenic Bcl-2 (9 and V. N. Ivanov, unpublished results).

The primary biochemical function of Bcl-2 is still unknown; however, it is highly likely that Bcl-2 interacts with the intracellular signaling pathways at several levels, because Bcl-2 can associate with Ras (38), recruit the Raf-1 kinase (39) and suppress apoptosis by the inhibition of endoplasmic reticulum-associated Ca^{2+} fluxes (40). These interactions of Bcl-2 with signaling pathways may result in modulation of TF (41). Suppressive effect of Bcl-2 on the proteasome activity during GR-mediated apoptosis may reflect a more general capacity of Bcl-2 for the negative regulation of protein degradation. Indeed, the caspase-dependent degradation of transcription factors AP-1, NUR-77 and NF-κB p50–p50 after Dex treatment were suppressed both by an irreversible peptide inhibitor of this protease family, zVAD-fmk, and partially or completely by the presence of various levels of the transgenic Bcl-2, consistent with a role Bcl-2 in the negative regulation of proteolysis (11,42,43). Suppression of the cleavage of the caspase-3 (CPP32) (44) and Mch3 (45) substrate PARP by transgenic Bcl-2 during the early apoptotic commitment also supports this suggestion. The substrate specificity of the various caspases that operate in the thymus is not completely known, however, the potential caspase cleavage site exists in the N-end of NF-κB p50; additional studies are necessary to functionally confirm the relevance of this site. Of note, caspase-dependent cleavage of SP-1 TF during retinoid-induced apoptosis was recently reported (46).

Although new gene expression is linked with the activation of caspase-dependent proteolysis following Dex treatment, the mechanism of this activation is unknown. While the proteasome was proposed to play a role in activating caspase-dependent proteolysis (47,48), the ultimate biochemical proof is still missing and additional studies are necessary to identify a GC-regulated activator of this proteolysis (or a negative regulator of its inhibitor). Results presented here indicate the involvement of the proteasome in early apoptotic commitment following Dex treatment and argue against the critical role of the proteasome in AICD. Early inhibition of Dex-induced apoptosis and late apoptosis induction by proteasome inhibitors by themselves can be

interpreted in several ways. First, it is possible that proteasome facilitates and accelerates Dex-induced apoptosis, and that its inhibition results only in a delay of apoptosis. This would implicate the existence of proteasome-dependent and -independent pathways of Dex-mediated apoptosis signaling. Second, the role of a proteasome in initiating Dex-induced apoptosis could be essential and all subsequent death observed with Dex lactacystin could be due no longer to Dex, but rather to lactacystin. In this case, one would have to speculate that an apoptosis activator, perhaps even an activated caspase, that is normally efficiently degraded by proteasome, accumulates during the later phases of proteasome inhibition (12 h) and then initiates apoptosis. At present, the fact that proteasome inhibitors caused DP apoptosis by themselves upon prolonged incubation precludes the discrimination between these possibilities.

By contrast to Dex stimulation, the early events of intracellular signaling during PMA- and/or ionomycin-induced death *ex vivo* and *in vivo* were followed by an induction/activation of transcription factors AP-1 (Jun–Fos), NUR-77 and NF-κB, that is reminiscent of the early phase of activation-induced apoptosis of mature T cells (34). We observed that irreversible commitment to TCR agonist-induced apoptosis required continuous exposure of DP thymocytes to these stimuli, in contrast to Dex, which acted almost instantaneously (Figs 1 and 3, and not shown). This is consistent with the proposed primary/secondary signaling cascades in AICD, whereby TCR signaling would induce cell surface expression of the TNF/NGF family of ligands, whose interaction with cognate receptors would result in the activation of death signaling, akin to the AICD of mature T cells and T cell hybridomas (reviewed in 34). To date, however, the existence of the secondary cascade has not been confirmed and the exact players in the secondary cascade remain unknown.

Our results show that nuclear TF are cleaved in Dex-induced apoptosis, that this cleavage is caspase-dependent and that it precedes DNA fragmentation. However, the specific role of TF regulation in cell death and survival remains unclear. We and others have recently observed substantial increase in DP thymocyte apoptosis from *fos*$^{-/-}$ mice in cases of Dex-induced apoptosis and AICD of thymocytes (13), and for the UV-induced apoptosis of fibroblasts of these mice (49). These results suggest an AP-1/Fos-dependent regulation of 'survival' genes (7) following apoptotic signaling. Caspase-dependent cleavage of AP-1 during apoptotic commitment of DP thymocytes following Dex stimulation (results of this study and 50) would be consistent with the idea (13,49,50) that AP-1 might be involved in the transcriptional control of survival genes. Another candidate for the control of cell survival might be the RelA-p50 induced after Dex treatment of thymocytes, as was observed in this study. In several recent publications, an essential role of NF-κB/RelA in preventing TNF-induced death was established in *RelA*$^{-/-}$ cells or by inhibition of RelA in normal cells (51–54). A substantial role of NF-κB in thymocyte development, especially for anti-apoptotic protection during positive selection, was also described (22,55–59). Although the nature of the gene products regulating cell survival is still unknown, protein inhibitors of caspases or enzymes degrading caspases would be good candidates. Owing to the poor ability of DP cells to be transfected, at present, we cannot conclusively distinguish whether TF degradation is causally linked to Dex-induced apoptosis or whether this is an epiphenomenon. Resolution of this question will have to await the construction of caspase-resistant TF transgenes.

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Abbreviations

AICD	activation-induced cell death
AP-1	activator protein-1
CREB	cyclic AMP-response-element-binding protein
Dex	dexamethasone
DP	double-positive
EMSA	electrophoretic mobility shift assay
GC	glucocorticoid
GR	glucocorticoid receptor
LLnL	*N*-acetyl-Leu-Leu-norleucinaldehyde
NGF	nerve growth factor
NF-κB	nuclear factor κB
PARP	poly(ADP-ribose) polymerase
PI	propidium iodide
PMA	phorbol myristate acetate
TF	transcription factor
zVAD-fmk	benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

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