

## Caspase-Mediated Calcineurin Activation Contributes to IL-2 Release during T Cell Activation

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**Calcineurin, a  $\text{Ca}^{2+}$ /calmodulin-dependent Ser/Thr phosphatase (protein phosphatase 2B), plays a critical role in IL-2 production during T cell activation. It has been previously reported that IL-2 release in activated Jurkat T requires caspase-like activity (Posmantur *et al.* (1998) *Exp. Cell. Res.* 244, 302–309). We report here that the 60-kDa catalytic subunit of calcineurin A (Cn A) was partially cleaved to a 45-kDa form in phytohemagglutinin A (PHA) or phorbol ester + ionomycin (P + I)-activated Jurkat cells. In parallel, proteolytic activation of upstream caspases (caspase-8 and -9) as well as effector caspase-3 was also observed. Cn A cleavage was caspase mediated, since it was inhibitable by pan-caspase inhibitor Cbz-Asp-CH<sub>2</sub>OC(O)-2,6-dichlorobenzene (Z-D-DCB). Cn A cleavage was also observed when purified calcineurin was digested *in vitro* with caspase-3. Truncated Cn A was associated with enhanced phosphatase activity and reduced calmodulin sensitivity. Furthermore, in PHA or P + I-activated Jurkat cells, dephosphorylation of calcineurin substrate NFATc (a transcription factor known to be involved in transactivation of the IL-2 gene), was also suppressed by Z-D-DCB. Taken together, our results suggest that caspase-mediated cleavage of Cn A contributes to IL-2 production during T cell activation.** © 2001 Academic Press

**Key Words:** calcineurin; protein phosphatase; caspase; NFAT; IL-2; T cell activation.

Abbreviations used: PKC, protein kinase C; MAPK, mitogen-activated protein kinase; PHA, phytohemagglutinin A; PMA, phorbol 12-myristate 13-acetate; I, ionomycin; CaM, calmodulin; Cn A, calcineurin A; CsA, cyclosporine A; PARP, poly(ADP-ribose) polymerase; Z-D-DCB, Cbz-Asp-CH<sub>2</sub>OC(O)-2,6-dichlorobenzene; NFAT, nuclear factor of activated T cells and DTT, dithiothreitol.

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Antigen presentation in context with MHC molecules results in the physiological activation of T cells (1, 2). During T cell activation, signaling events are triggered that lead to production of hematopoietic growth factors such as IL-2, IL-3, and GM-CSF. IL-2 is necessary for T cell growth and clonal expansion. Among the early T cell signaling events are activation of protein tyrosine kinases (fyn, ZAP-70, lck), phosphatidyl inositol metabolism and increases in the levels of intracellular calcium. These events lead to activation of PKC/MAPK pathways as well as activation of protein phosphatase 2B (calcineurin). Activation of calcineurin causes dephosphorylation and nuclear translocation of NFAT, a transcription factor critical for IL-2 production (3). NFAT, once inside the nucleus, forms DNA binding complexes by cooperatively interacting with AP-1 family members (Fos/Jun) and transactivates cytokine genes such as IL-2. Thus calcineurin plays a key role in T cell activation.

Calcineurin is a highly conserved  $\text{Ca}^{2+}$ /calmodulin (CaM) dependent Ser/Thr phosphatase (4). The heterodimeric molecule of calcineurin consists of a 60-kDa catalytic subunit (Cn A) and a 19-kDa  $\text{Ca}^{+2}$  binding regulatory subunit (Cn B). Calcineurin is the molecular target of immunosuppressive agents, cyclosporine A (CsA) and FK506 (4, 5). NFAT, a substrate of calcineurin, was originally described to be a transcription factor critical for IL-2 expression. NFAT binding sites have since been reported to be present within the promoter regions of other cytokines including IL-3, GM-CSF and TNF $\alpha$  as well as cell surface receptors such as CD40L and Fas L (3). NFAT protein family consists of several isoforms; NFATp (NFAT1 or NFATc2), NFATc (NFAT2 or NFATc1), NFAT3 (NFATc4), NFAT4 (NFATx or NFATc3) and a very recently identified isoform NFAT5 (6). NFATp and NFATc have been shown to strongly activate IL-2 and IL-4 promoters (3). All known isoforms of NFAT are activated by calcineurin, except NFAT5, which is constitutively present inside the nucleus. Studies on inactivation of

NFATp, NFATc and NFAT4 genes in mice suggest that NFAT plays an important role in an effective, regulated immune response (7–14).

It has been shown previously that activation of Jurkat T cells with PHA causes activation of caspase-3 and a pan-caspase inhibitor Z-D-DCB inhibits IL-2 release (15). The role of caspase-3 in apoptosis is well established (16, 17); however there are reports suggesting that caspase-3 activation can also occur under non-apoptotic conditions (18, 19). Under apoptotic conditions, activation of upstream caspases (caspase-8 and 9) proteolytically activates downstream effector caspases such as caspase-3. During proteolytic activation of caspase-3 (20), the 32-kDa caspase-3 precursor polypeptide undergoes proteolytic processing by caspase-8 or caspase-9 to 20- and 12-kDa polypeptides. The 20-kDa polypeptide undergoes further autolysis to give rise to 17-kDa polypeptide. The p17 and p12 heterodimeric complex is the mature form of caspase-3. Activated caspase-3 can cleave a number of intracellular proteins including cytoskeletal protein  $\alpha$ -spectrin II and poly(ADP-ribose) polymerase (PARP).

To better understand the role of caspase in IL-2 release during Jurkat T cell activation, we decided to investigate the potential caspase substrate(s) involved in T cell activation. We report here that in PHA or PMA + I activated Jurkat T cells, the 60-kDa catalytic subunit of calcineurin (Cn A) is cleaved to a 45-kDa form by caspase. Cleavage of Cn A by caspase-3 augments its phosphatase activity and reduces its sensitivity to calmodulin. Furthermore, pan caspase inhibitor Z-D-DCB partially inhibits calcineurin-mediated dephosphorylation of NFATc in activated Jurkat cells. Collectively, these data show, for the first time, that during Jurkat T cell activation, caspase-mediated proteolytic activation of calcineurin A contributes to IL-2 production.

## EXPERIMENTAL PROCEDURES

**Materials.** Purified active bovine brain calcineurin (C1907) and *p*-nitrophenyl phosphate was purchased from Sigma (St. Louis, MO). Purified active recombinant human caspase-3 (66281T), anti-human caspase-3 polyclonal antibody (65906E) and anti human caspase-7 monoclonal antibody (66871A) were from Pharmingen (San Diego CA). Anti-calcineurin monoclonal antibody (C26920) was from Transduction Labs (Lexington, KY), monoclonal anti-NFATc antibody (MA3-024; clone 7A6) was from Affinity Bioreagents (Golden, CO) and anti- $\alpha$ -spectrin II antibody (FG 6090; clone AA6) was from Affinity Research Products (Nottingham, UK). Anti-human Fas antibody (05-201; clone CH11) was purchased from Upstate Biotech. (Lake Placid, NY). Anti-human caspase-8 antibody (sc-7890) and anti-human caspase-9 (sc-7885) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-human XIAP antibody (AF822) was from R&D systems, Inc. (Minneapolis, MN). PHA was from DIFCO Laboratories (Detroit, MI) and PMA and ionomycin were purchased from Calbiochem (La Jolla, CA). All reagents for cell culture were obtained from Gibco-BRL (Grand Island, NY).

**Cell culture and cell stimulation.** All experiments were performed on Jurkat, a T cell leukemia cell line. Jurkat cells (clone E6-1,

ATCC TIB 152, American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin at 37°C in the presence of 5% CO<sub>2</sub>. Both for IL-2 production and Western blotting, incubations with PHA were always performed in serum free media. For PHA stimulation, Jurkat cells were washed in serum-free RPMI media three times prior to incubation with PHA. Incubations with PMA + I was always performed in the presence of RPMI media containing serum.

**Protein extraction and Western blotting.** For Western blotting, typically  $10 \times 10^6$  cells were stimulated with PHA (5  $\mu$ g/ml) or PMA (1.25 ng/ml) + I (1  $\mu$ M) in 5 ml medium; in experiments where pan-caspase inhibitor Z-D-DCB (Z-Asp-CH<sub>2</sub>OC(O)-2,6-dichlorobenzene; 100  $\mu$ M), calcineurin inhibitor cyclosporine A (1  $\mu$ M) or calpain inhibitor (MDL 28170, 30  $\mu$ M) were included, cells were preincubated in the presence of the inhibitor at 37°C for 1 h prior to addition of the stimulants. The concentrations chosen for each of these inhibitors have been shown previously to be effective in inhibiting caspase-3 (15), calcineurin (5) and calpain (21), respectively. Following stimulation, cells were collected, washed with cold PBS and total cellular proteins were extracted in Triton X-100 buffer (22). Protein concentration was determined according to a modified Lowry procedure (Bio-Rad D-C protein assay). For Western blotting, 20–35  $\mu$ g of total cellular protein was subjected to SDS/PAGE using 4–20% acrylamide gradient gels (Novex, San Diego, CA). Following electrophoresis, proteins were transferred to PVDF (polyvinylidene difluoride, 0.2  $\mu$ m pore size) membranes, blocked with 5% nonfat milk and immunoblotted with the desired antibody. The blots were developed employing alkaline phosphatase reaction. Anti-NFATc antibody (clone 7A6, Affinity Bioreagents) was generated against a recombinant GST-fusion protein including residues 1–654 of NFATc and is specific for NFATc (23). This antibody has been shown to immunoreact with NFATc in peripheral blood T cells as multiple bands ranging from 80 to 140 kDa, representing different NFATc isoforms [due to alternate splice/polyadenylation events; (24–26)] and their differentially phosphorylated forms. Two anti-caspase-3 antibodies were used in this study. One of the anti-caspase-3 antibodies used in this study immunoreacted with 32-kDa caspase-3 precursor protein and proteolytically generated p20 and p17 fragments but not the p12 fragment. The other anti-caspase-3 antibody used in this study immunoreacted with the 32-kDa precursor protein and the proteolytically generated 12-kDa subunit of caspase-3. Anti-caspase-8 antibody immunoreacted with the 55-kDa precursor protein and 44/42-kDa proteolytic fragments generated during activation. Anti-caspase-9 antibody immunoreacted with 45-kDa precursor and 35-kDa proteolytically generated fragments. Anti-XIAP antibody immunoreacted with intact XIAP and a 30-kDa proteolytically generated fragment of XIAP. Anti-calcineurin A antibody (Transduction labs) was generated against human calcineurin (residues 247–449) and it immunoreacted with the 60-kDa calcineurin A subunit (Cn A).

**Measurement of IL-2 released in Jurkat cell conditioned medium.** For IL-2 measurement, Jurkat cells ( $2 \times 10^6$ ) were incubated in 2 ml medium and stimulated with PHA (5  $\mu$ g/ml) or PMA (1.25 ng/ml) + I (1  $\mu$ M) for 18 h. IL-2 released by untreated or treated Jurkat cells was measured in the cell conditioned culture medium by human IL-2 ELISA kit according to the manufacturer's procedure [R&D Systems, Minneapolis, MN (15)].

**In vitro digestion of calcineurin with recombinant caspase-3 and measurement of calcineurin activity.** Purified bovine brain calcineurin (1.5  $\mu$ g) was digested with recombinant caspase-3 (3  $\mu$ g) in the presence of 100 mM Tris-Cl; pH 7.5, 10 mM DTT, 10% glycerol, 100  $\mu$ M EGTA in a final volume of 165  $\mu$ l at room temperature for 2 and 5 h. Control incubation contained calcineurin in the absence of caspase-3. Following incubation, 50  $\mu$ l (0.45  $\mu$ g) of the digestion mixture was assayed for calcineurin activity by measuring the hydrolysis of *p*-nitrophenyl phosphate at 405 nm as previously described (27). Briefly, the assay buffer consisted of 50 mM Tris-Cl (pH

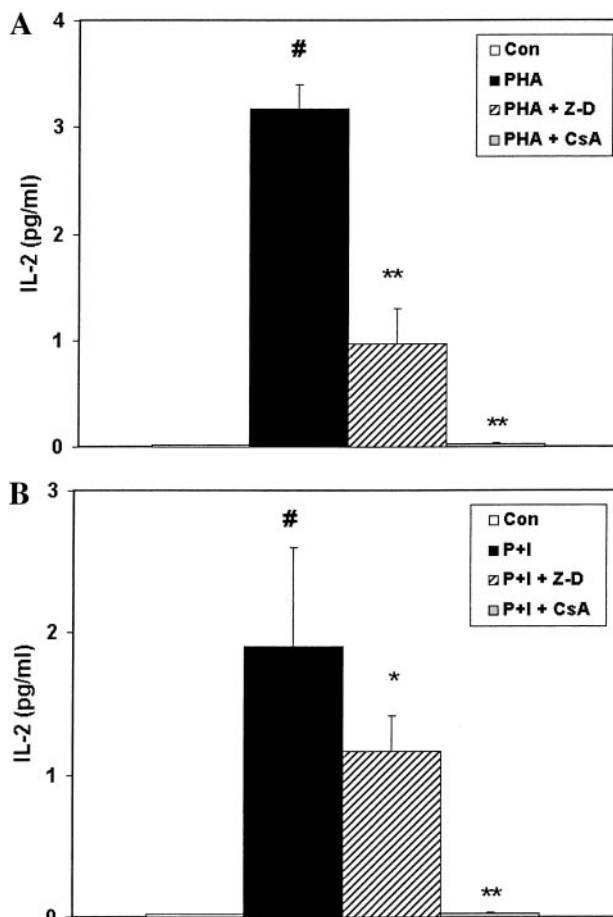
7.5), 2.5 mM DTT, 20 mM *p*-nitrophenyl phosphate and one of the following: (i) 1 mM EDTA for measurement of background activity, (ii) 100 nM EDTA + 1 mM Ca<sup>2+</sup> + 1 mM Mn<sup>2+</sup> for basal activity, and (iii) 100 nM EDTA + 1 mM Ca<sup>2+</sup> + 1 mM Mn<sup>2+</sup> + 0.8 μM calmodulin (CaM) for CaM-stimulated activity. Since calcineurin activity is enhanced in the presence of Mn<sup>2+</sup> (27), therefore for ease of detection, Mn<sup>2+</sup> was included in the assay buffer for conditions (ii) and (iii). For quantification, the background activity in the presence of 1 mM EDTA was subtracted from the Ca<sup>2+</sup>/Mn<sup>2+</sup> and Ca<sup>2+</sup>/Mn<sup>2+</sup>/CaM activity and then plotted. The ratio of CaM-stimulated calcineurin activity over basal activity will be referred to as fold stimulation by CaM.

## RESULTS

*Comparison of pan-caspase inhibitor Z-D-DCB and calcineurin inhibitor cyclosporine A on IL-2 release from activated Jurkat T cells.* It has been shown previously that IL-2 released during activation of Jurkat cells by PHA is inhibited by a pan caspase inhibitor Z-D-DCB (15). To compare and quantify the contribution of calcineurin and caspase toward IL-2 release, Jurkat cells were activated with two T cell activating agents; PHA or PMA + ionomycin (PMA + I). Incubation with PHA was performed in serum-free media. When PMA + I was used for stimulation, incubations were performed in the presence of media containing serum because significant amounts of IL-2 was released only when serum was included in the media.

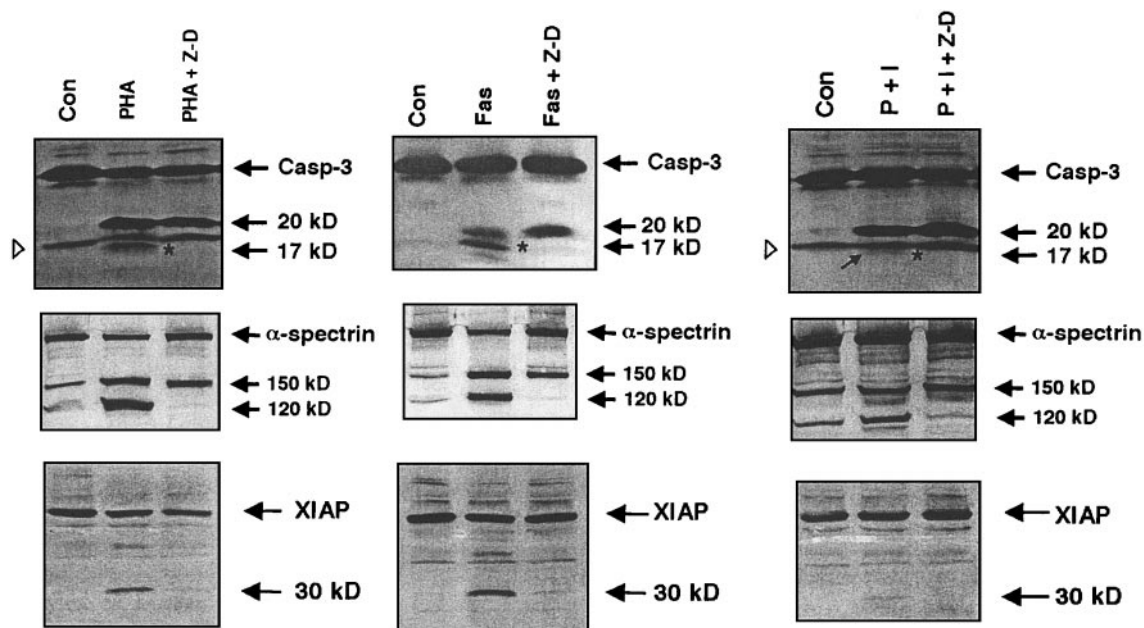
Upon PHA stimulation, Jurkat cells released significant amounts of IL-2 detected by ELISA which was inhibited almost completely (99%) in the presence of cyclosporine A (1 μM) (Fig. 1A). IL-2 release was also inhibited (70%) by Z-D-DCB (100 μM). Upon stimulation with PMA + I (Fig. 1B), IL-2 release was again inhibited almost completely by cyclosporine A (99%) and partially by Z-D-DCB (37%). We have further repeated these experiments on separate days with different cell preparations and obtained similar results. Taking all the experiments together, the inhibition of IL-2 release by Z-D-DCB ranged from 70 to 92% upon PHA stimulation and 21–57% upon PMA + I stimulation. These results indicate that pan caspase inhibitor Z-D-DCB inhibited, at least partially, both PHA and PMA + I stimulated IL-2 release in Jurkat cells and the inhibition appeared to be greater when cells were stimulated with PHA than with PMA + I.

*Proteolytic processing and activation of caspase-3 in activated Jurkat T cells.* During activation, the 32-kDa precursor caspase-3 is proteolytically cleaved to form p20 and p12 by upstream caspases such as caspase-8 and -9. Further proteolytic cleavage of p20 results in formation of p17 fragment. The p17/p12 dimer is known to have catalytic activity. In PHA activated Jurkat cells, the 32-kDa caspase-3 precursor was proteolytically cleaved to p20 and p17 fragments (Fig. 2, top panel). Upon careful examination of the immunoblot, it appears that the 32-kDa caspase-3 precursor was proteolytically processed to a large amount



**FIG. 1.** Inhibition of IL-2 release by Z-D-DCB in PHA or PMA + I-activated Jurkat cells. Release of IL-2 into the cell culture medium by Jurkat cells stimulated for 18 h with PHA (5 μg/ml) in medium without serum or PMA + I (PMA, 1.25 ng/ml; I, 1 μM) in medium without serum was measured by ELISA. In A, untreated cells (Con, open bar), PHA-stimulated cells without (PHA, solid bar) or with 100 μM Z-D-DCB (PHA + Z-D, crossed bar) or 1 μM CsA (PHA + CsA, shaded bar). In B, untreated cells (Con, open bar), PMA + I-stimulated cells without (P + I, closed bar) or with Z-D-DCB (P + I + Z-D, crossed bar) or 1 μM CsA (P + I + CsA, shaded bar). Data are mean ± SEM (*n* = 3). Those values different from control are indicated by # (*P* < 0.05, Student *t* test) and values different from PHA (A) or P + I (B) alone are indicated by \* (*P* < 0.05) or \*\* (*P* < 0.01).

of p20 fragment. Yet, only a small amount of p20 was further processed to p17 (marked by an asterisk in Fig. 2). In contrast, conversion of p20 to p17 was inhibited in the presence of 100 μM Z-D-DCB. Upon PMA + I stimulation (Fig. 2), caspase-3 was processed to p20 at levels comparable to PHA treatment but further conversion to p17 was very mild (see asterisk in Fig. 2) when compared with PHA treatment (Fig. 2). Once again conversion of p20 to p17 was inhibited in the presence of Z-D-DCB. In contrast, Anti-Fas treatment induces a large accumulation of p17 fragment. It has been shown previously Since 32-kDa precursor caspase-3 upon proteolytic activation forms p20 and



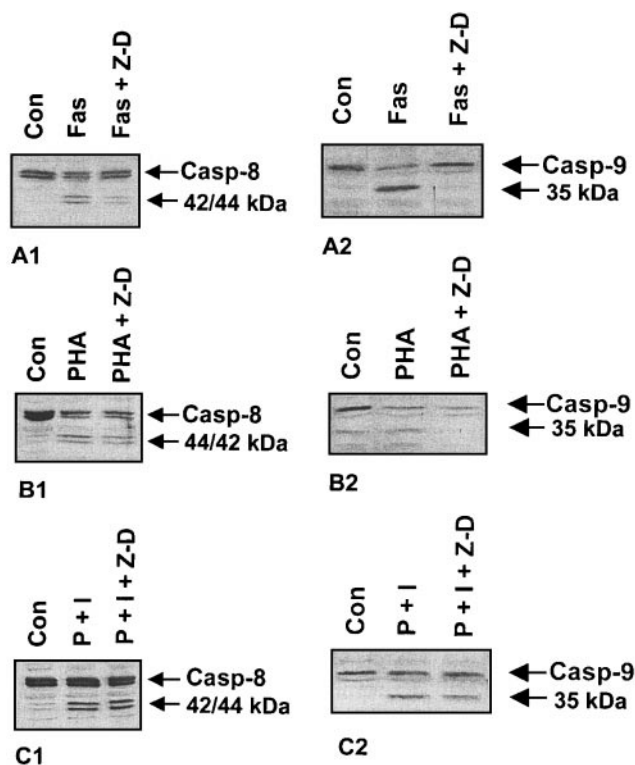
**FIG. 2.** Caspase-3 activation and proteolytic cleavage of caspase-3 substrates under activating or apoptotic conditions. Jurkat cells were treated with PHA (5  $\mu\text{g}/\text{ml}$ ; left panel) for 24 h and for comparison, with anti-Fas antibody (500 ng/ml; middle panel) for 5 h (middle panel) or with PMA + I (PMA, 1.25 ng/ml; I, 1  $\mu\text{M}$ ) (right panel). Incubations were done in the absence (con) or presence of Z-D-DCB (PHA + Z-D or Fas + Z-D or P + I + Z-D). Following treatment, total cellular proteins were analyzed on SDS-PAGE and immunoblotted for caspase-3 (32 kDa) (top) and  $\alpha$ -spectrin II (280 kDa) (middle) or XIAP (bottom). The asterisk in the figures indicates the 17-kDa caspase-3 proteolytic fragment. Solid arrows indicated the intact proteins of their proteolytic fragments. The open arrow on the left indicates either a band unrelated to caspase-3 or a nonspecifically generated caspase-3 fragment. Results are representative of three separate experiments.

p12, cell lysates from PHA or PMA + I or anti-Fas-treated cells were blotted for p12. Under these conditions, significant amounts of p12 were formed (data not shown). A band about 19 kDa was sometime observed both in untreated and activated cells. It is either a protein unrelated to caspase-3 or a nonspecifically generated caspase-3 fragment.

**Proteolytic cleavage of caspase-3 substrates:  $\alpha$ -spectrin II and X-linked inhibitor of apoptosis protein (XIAP).** Caspase-3-mediated proteolytic cleavage of  $\alpha$ -spectrin II, as judged by the loss of intact  $\alpha$ -spectrin II was also greater with anti-Fas challenge than with PHA treatment, consistent with our previous report (15). Upon PHA treatment both 150 and 120-kDa  $\alpha$ -spectrin II breakdown products, characteristic of caspase-3-mediated proteolysis, were formed (Fig. 2, middle panel). Formation of 120 kDa product was inhibited with Z-D-DCB. Integrity of another caspase substrate XIAP in both PHA or P + I-activated or apoptotic Jurkat cells was also examined (Fig. 2, bottom panel). Again, XIAP was significantly fragmented to a 30-kDa form in both PHA and anti-Fas treatment. P + I treatment also induced XIAP fragmentation, but to a lesser extent. Since XIAP protein is an endogenous caspase inhibitor (28), its fragmentation could contribute to the increased caspase-3 activity in activated Jurkat cells.

**Caspase-8 and -9 activation in activated Jurkat cells.** To understand how caspase-3 is activated in PHA and P + I-activated Jurkat cells, we examined the integrity of the upstream caspases (caspase-8 and -9) that are known to activate caspase-3 by the receptor-mediated and mitochondrial mediated pathways, respectively (16). For comparison, Jurkat cells were also treated with anti-Fas to induce apoptosis. As expected, anti-Fas treatment induced caspase-8 processing and activation (Fig. 3). Caspase-9 was also processed and activated. Z-D-DCB was effective in blocking both caspase-8 and -9 processing here (Fig. 3). Interestingly, in PHA-treated cells, caspase-8 was significantly processed and activated. In the case of caspase-9, intact protein was reduced by PHA-treatment, but its activated form only mildly accumulated (Fig. 3). In P + I treatment, both caspase-8 and -9 were processed and activated. Under these conditions, Z-D-DCB was not very effective in inhibiting caspase-8 and -9 processing.

**Proteolytic processing of calcineurin by caspase-3 in PHA and PMA + I-activated Jurkat T cells.** Calmodulin-binding proteins, such as  $\text{Ca}^{2+}/\text{CaM}$ -dependent protein kinase IV,  $\text{CaM}$ -dependent kinase kinase,  $\alpha$ II-spectrin II are prone to proteolysis both by caspase and calpain (29, 30). Since calcineurin is a  $\text{CaM}$ -dependent enzyme and is involved in T cell activation, we investigated whether calcineurin was proteolyti-



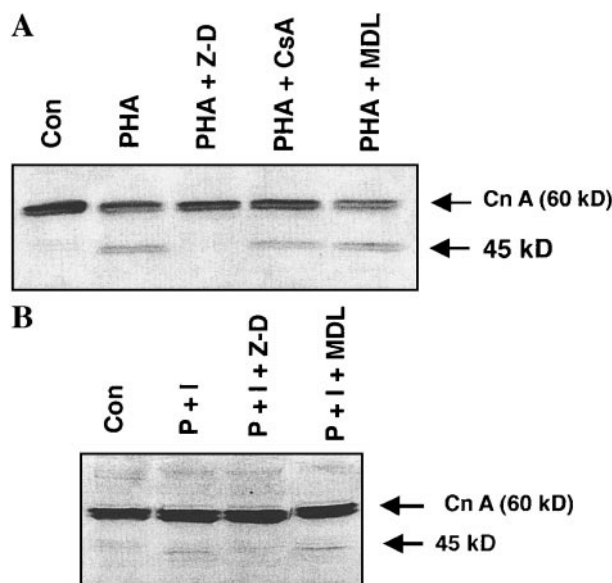
**FIG. 3.** Caspase-8 and -9 activation under activating or apoptotic condition in Jurkat cells. In A, Jurkat cells were either untreated (Con) or treated with anti-Fas in the absence and presence of Z-D-DCB (100  $\mu$ M) for 24 h. In B Jurkat cells were either untreated (Con) or treated with PHA in the absence and presence of Z-D-DCB (100  $\mu$ M) for 24 h. In C, Jurkat cells were treated with P + I in the absence and presence of Z-D-DCB (100  $\mu$ M) for 24 h. Total cellular proteins (20  $\mu$ g) were subjected to SDS-PAGE and analyzed by Western blotting using anti-caspase-8 (left panels) or anti-caspase-9 (right panels) antibodies. Results are representative of two separate experiments.

cally cleaved during Jurkat T cell activation. Unactivated as well as PHA-activated Jurkat cells showed a prominent band around 60 kDa (Fig. 4A) corresponding to the A subunit of calcineurin (Cn A). With PHA treatment, the intact Cn A was partially converted to a fragment around 45 kDa (Fig. 4A). Formation of the 45-kDa band was inhibitable by Z-D-DCB but not by cyclosporine A or calpain inhibitor MDL28170, suggesting that its formation was mediated by caspase(s). When Jurkat cells were stimulated with PMA + I (Fig. 4B), the cleavage of intact Cn A to 45-kDa fragment was significantly less; only a very small amount of Cn A was cleaved. Thus the extent of Cn A cleavage was dependent upon the activation stimuli used.

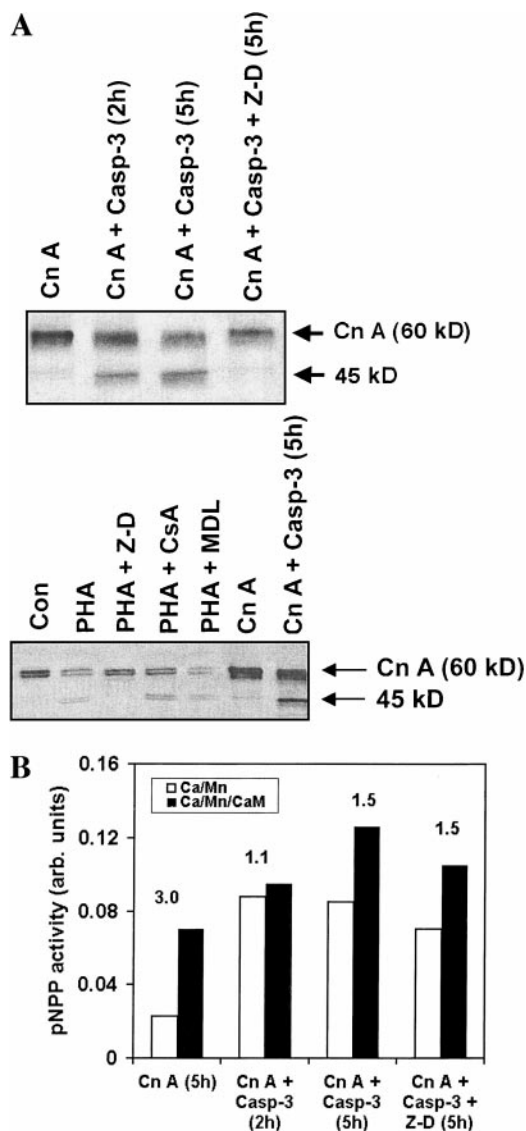
*Purified calcineurin is proteolytically activated in vitro by caspase-3.* To examine if calcineurin, like other CaM-binding proteins, is a direct caspase-3 substrate, purified bovine calcineurin was digested *in vitro* with recombinant human caspase-3 for 2 and 5 h. As a control, calcineurin was incubated without caspase-3. Digestion of calcineurin by caspase-3 for both 2 and 5 h

resulted in formation of the 45-kDa fragment which was inhibitable by Z-D-DCB (Fig. 5A, top panel), confirming our results of the cell based experiments. For comparison, protein samples from Jurkat cells treated with PHA (5  $\mu$ g/ml in the absence and presence of Z-D-DCB (100  $\mu$ M), CsA (1  $\mu$ M) or MDL28170 (30  $\mu$ M) for 24 h were run along side with untreated or caspase-3 digested calcineurin (Fig. 5A, bottom panel). We found that the 45-kDa Cn A fragment generated in activated Jurkat cells co-migrated with the 45-kDa fragment generated *in vitro* by caspase-digestion.

We then asked the question whether cleavage of calcineurin A caused increase in its phosphatase activity. To address this question, aliquots of the *in vitro* digestion mixture was assayed for calcineurin activity using p-nitrophenyl phosphate as a calcineurin substrate. With increasing time of digestion of calcineurin with caspase-3, there was enhancement of both basal and CaM stimulated calcineurin activity (Fig. 5B), concomitant with the accumulation of 45-kDa fragment (Fig. 3A). Additionally, there was a loss of CaM sensitivity of calcineurin (the numbers above the bars indicate the fold stimulation by calmodulin (Fig. 5B)). The presence of Z-D-DCB partially inhibited the caspase-mediated calcineurin activation. Thus our cell-based and *in vitro* digestion data clearly demonstrated that calcineurin A is a caspase-3 substrate, joining the rank of an increasing number of CaM-binding proteins as caspase substrates (31).



**FIG. 4.** Caspase-3 proteolytically cleaves calcineurin in activated Jurkat cells. Jurkat cells were treated with PHA (5  $\mu$ g/ml) (A) and PMA + I (PMA, 1.25 ng/ml; I, 1  $\mu$ M) (B) in the absence and presence of Z-D-DCB (100  $\mu$ M), CsA (1  $\mu$ M), or MDL28170 (30  $\mu$ M) for 24 h. Total cellular proteins (20  $\mu$ g) were subjected to SDS-PAGE and analyzed by Western blotting using anti-calcineurin A antibody. The arrow indicated intact calcineurin A (60 kDa) and its major fragment (45 kDa). Results are representative of three separate experiments.

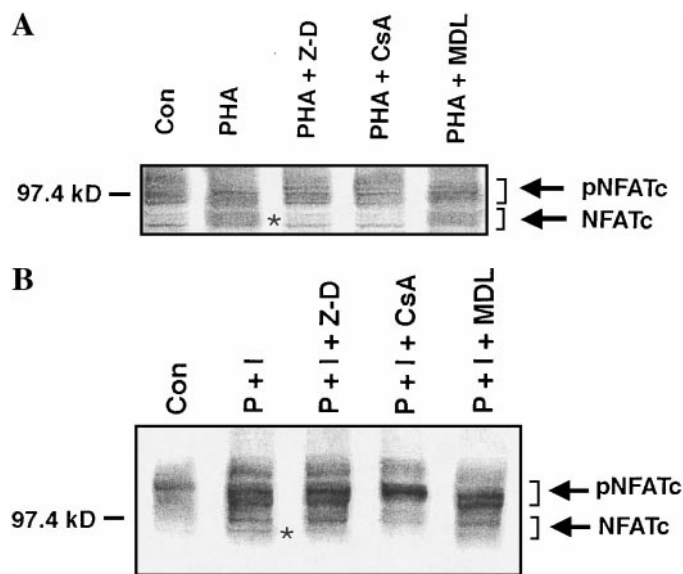


**FIG. 5.** Caspase-3 proteolytically cleaves calcineurin A and enhances its phosphatase activity *in vitro*. In A and B purified bovine calcineurin A was incubated with recombinant human caspase-3 for 2 and 5 h (as described under Materials and Methods). Following digestion, 1  $\mu$ l (9 ng) of the digestion mixture was subjected to SDS-PAGE and immunoblotted with anti-calcineurin A antibody (A, top panel); and 50  $\mu$ l of the digestion mixture (0.45  $\mu$ g) was assayed for calcineurin activity using *p*-nitrophenyl phosphate as a substrate (B). The basal (Ca<sup>2+</sup>/Mn<sup>2+</sup>; open bars) and CaM-stimulated activities (Ca<sup>2+</sup>/Mn<sup>2+</sup>/CaM; solid bars) are shown. The numbers (in brackets) above each set of bars indicate the fold-stimulation of calcineurin activity by calmodulin. In A (bottom panel), for comparison, protein samples from Jurkat cells treated with PHA (5  $\mu$ g/ml in the absence and presence of Z-D-DCB (100  $\mu$ M), CsA (1  $\mu$ M), or MDL28170 (30  $\mu$ M) for 24 h were run along side with untreated or caspase-3-digested calcineurin (right two lanes). Blot was analyzed using anti-calcineurin A antibody. Results are representative of two separate experiments.

*Dephosphorylation of NFATc in PHA and PMA + I-activated Jurkat cells is inhibited by Z-D-DCB.* To investigate whether caspase-mediated proteolytic activation of calcineurin in Jurkat T cells contributes to

dephosphorylation of transcription factor NFATc, we investigated the phosphorylation status of NFATc in Jurkat cells stimulated with PHA or PMA + I, in the presence and absence of Z-D-DCB. In untreated cells, we detected multiple bands around 100 kDa and above (Fig. 6A) corresponding to the phosphorylated isoforms of NFATc (pNFATc) as reported previously (23–26). Upon PHA stimulation, as expected, we observed the appearance of multiple faster-migrating bands representing dephosphorylated forms of NFATc (marked by asterisk, Fig. 6A). In PHA-stimulated cells treated with Z-D-DCB, the dephosphorylation of NFATc was inhibited to a similar extent as cyclosporine A (Fig. 6A). Calpain inhibitor MDL28170 had no effect on dephosphorylation of NFATc.

Upon PMA + I treatment, once again the faster-migrating dephosphorylated NFATc forms were observed (Fig. 6B). In addition, the intensities of all NFATc bands were increased, most likely due to induction of NFATc upon activation as reported previously (3, 24). The appearance of dephosphorylated forms of NFATc were drastically inhibited by cyclosporine A. Caspase inhibitor Z-D-DCB was most effective in inhibiting the appearance of only the fastest migrating



**FIG. 6.** Dephosphorylation of NFATc is inhibited by Z-D-DCB in PHA or PMA + I-activated Jurkat cells. Jurkat cells were stimulated with (A) PHA (5  $\mu$ g/ml) or (B) PMA + I (PMA, 1.25 ng/ml; I, 1  $\mu$ M) for 24 h. Total cellular proteins were analyzed by SDS-PAGE and immunoblotted with anti-NFATc antibody. In A the lanes correspond to untreated cells (con), PHA-treated cells without (PHA), or with 100  $\mu$ M Z-D-DCB (PHA + Z-D), 1  $\mu$ M CsA (PHA + CsA), or 30  $\mu$ M MDL28170 (PHA + MDL). In B the lanes correspond to untreated cells (con), PMA + I-treated cells without (P + I) or with 100  $\mu$ M Z-D-DCB (P + I + Z-D) or 1  $\mu$ M CsA (P + I + CsA) or 30  $\mu$ M MDL28170 (P + I + MDL). The phosphorylated NFATc bands are labeled as pNFATc and the dephosphorylated NFATc bands as NFATc. The position of the asterisk marks the dephosphorylated NFATc band(s). Results are representative of three separate experiments.

band (Fig. 6B, see asterisk) within the multiplet, indicating that the effect of Z-D-DCB was less dramatic with PMA + I than with PHA treatment. Again, MDL28170 had no effect on dephosphorylation of NFATc.

## DISCUSSION

Our group previously reported that IL-2 release in PHA activated Jurkat T cells is inhibited by pan-caspase inhibitor Z-D-DCB (15). In the present studies, we further investigated the mechanism of inhibition of IL-2 release by Z-D-DCB in PHA as well as P + I activated Jurkat cells. Interestingly, we observed that the 60-kDa catalytic subunit of calcineurin A (Cn A) was partially proteolytically cleaved to a shorter 45-kDa form in PHA or P + I activated Jurkat cells (Fig. 5). Interestingly, the fragmentation of Cn A paralleled the fragmentation of other caspase substrates, such as alpha-spectrin II and XIAP (Fig. 2). In addition, proteolytic activation of upstream caspases (caspase-8 and -9) (Fig. 3) as well as effector caspase-3 (Fig. 2) and caspase-7 (data not shown) was also observed, despite that no measurable programmed cell death was occurring within the time frame of T-cell activation we examined. Our suspicion that Cn A cleavage was mediated by effector caspase(s) was confirmed by the observation that (i) in Jurkat cell activation, Cn A fragmentation was inhibitable by Z-D-DCB (Fig. 4), and (ii) Cn A cleavage to 45-kDa fragment was reproduced with *in vitro* digestion with purified caspase-3 (Fig. 5). Importantly, *in vitro* Cn A fragmentation was concomitant with increase of phosphatase activity and decrease of CaM sensitivity (Fig. 5B), suggesting a deregulation of calcineurin activity control. We also attempted to measure calcineurin activity by immunoprecipitation upon Jurkat cell activation. Unfortunately, very little calcineurin was recovered by this method, disallowing us to monitor its activity (results not shown). But since both *in vitro* calcineurin digestion and *in situ* fragmentation of calcineurin during T-cell activation yielded antigenic fragments of identical size (Fig. 5A, bottom panel), it is reasonable to assume that calcineurin was at least in part activated proteolytically upon T-cell activation.

Since NFATc, a transcription factor known to be involved in trans-activation of the IL-2 gene (3), is also a crucial endogenous substrate of calcineurin, we turned our attention to its phosphorylation status (Fig. 6). Indeed, we observed that in PHA-stimulated T-cells, the dephosphorylation of NFATc by calcineurin was inhibitable by Z-D-DCB to almost the same extent as by CsA (Fig. 6). On the other hand, dephosphorylation of NFATc in P + I stimulated T-cells was only partially inhibited by Z-D-DCB. These data are consistent with the fact that suppression of IL-2 release by Z-D-DCB was more severe with PHA-treatment than

with P + I treatment (Fig. 1). It has also previously been established that calcineurin activation during T-cell activation was regulated by  $Ca^{2+}$  and calmodulin (3, 24). Together with the present results, it is plausible that calcineurin activation is mediated by two alternative pathways (calmodulin-mediated and caspase-mediated pathways). Also, the relative contributions of these two activation pathways could vary dependent on the stimulus used (e.g., PHA vs P + I).

It is important to stress that our studies now clearly established that effector caspases (-3 and -7) (Fig. 2 and data not shown) are activated in a non-programmed cell death situation. We further showed that upon T-cell activation, both upstream caspases (-8 and -9) are processed and activated (Fig. 3). Interestingly, while caspase-8 was evenly processed with both PHA and P + I treatments, activated form of caspase-9 only significantly accumulated with P + I treatment but not with PHA treatment. Also, unlike with anti-Fas, treatment, Z-D-DBC appeared to be less effective in inhibiting caspase-8 and -9 processing (Fig. 4). It suggests that the effect of Z-D-DBC on IL-2 release is a due to direct inhibition of caspase-3 processing (Fig. 4). It is also of interest to point out that endogenous caspase inhibitor protein XIAP was also fragmented in PHA and P + I treatment. This event was probably mediated by caspase-8 and/or -9, as previously reported (28). XIAP fragmentation might contribute to further activation of effector caspases we observed in activated Jurkat cells.

As a whole our studies showed that both upstream caspases as well as effector caspases actively participate in T-cell activation by proteolytic activation of calcineurin, which in turn dephosphorylates NFATc and thus gene expression. Presumably, the effector caspases are somehow being turned off once sufficiently amount of calcineurin was activated, otherwise the T-cells will very likely undergo full-blown programmed cell death. Further studies in this area will be required to elucidate how effector caspases are regulated during T-cell activation. In summary, we report for the first time that during Jurkat cell activation, calcineurin A is proteolytically activated by caspase-3. Such proteolytic activation of calcineurin A contributes, at least in part, to the dephosphorylation of its key substrate NFATc. These new observations provide a biochemical mechanism for regulation of IL-2 release by caspase-3. The present studies also raise the intriguing possibility that inhibition of caspases might provide a novel therapeutic opportunity for immunosuppression.

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