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Article

Targeted Cleavage of Signaling Proteins by Caspase 3 Inhibits T Cell Receptor Signaling in Anergic T Cells

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SUMMARY

T cell receptor (TCR) engagement in the absence of costimulation induces the calcium-dependent upregulation of a program of gene expression that leads to the establishment of T cell anergy. *Casp3* is one of the genes activated during anergy induction. Here we show that caspase 3 is required for the induction of T cell unresponsiveness. Suboptimal T cell stimulation induced caspase 3 activation, which did not result in cell death. Furthermore, caspase 3-deficient T cells showed impaired responses to anergizing stimuli. In anergic T cells, activated caspase 3 associated to the plasma membrane, where it cleaved and inactivated proteins such as the Grb2-related adaptor downstream of shc (GADS) and the guanine-nucleotide exchange factor Vav1, causing a blockade in TCR signaling. Our results identify a role for caspase 3 in nonapoptotic T cells and support that caspase 3-dependent proteolytic inactivation of signaling proteins is essential to maintain T cell tolerance.

INTRODUCTION

Random rearrangements of the gene segments at the T cell receptor (TCR) locus allow recognition of foreign antigens but also generate dangerous self-reactive T cells. Many cells carrying self-reactive TCRs are eliminated during negative selection in the thymus; however, this process has limitations and additional mechanisms of tolerance are required in the periphery to limit autoimmunity. Anergy is induced in T cells by partial or suboptimal stimulation, which results in functional inactivation of self-reactive cells. Anergic T cells show profound defects in interleukin 2 (IL-2) production and proliferation (Schwartz, 2003).

Clonal anergy is induced upon engagement of the TCR in the absence of costimulatory signals (Jenkins and Schwartz, 1987; Quill and Schwartz, 1987). Anergizing stimuli cause a sustained increase in intracellular free calcium, which induces the calcineurin-mediated dephosphorylation and nuclear translocation of members of the nuclear factor of activated T cells (NFAT) family of transcription factors, in the absence of full activation of activator protein 1 (AP-1) complexes (Jain et al., 1993; Macian et al., 2001, 2002). NFAT proteins play a key role in the induction of tolerance in T cells by driving the expression of anergy-inducing genes (Macian et al., 2002). The specific expression of these genes is required to impose a state of functional unresponsiveness through different mechanisms. Anergizing stimuli upregulate the expression of at least three E3-ubiquitin ligases: the Casitas B-lineage lymphoma b (Cbl-b), the itchy E3 ubiquitin protein ligase homolog (Itch), and the gene related to anergy in lymphocytes (GRAIL) (Anandasabapathy et al., 2003; Heissmeyer et al., 2004; Jeon et al., 2004), with a role in the downregulation of TCR signaling by inactivation or degradation of signaling molecules (Mueller, 2004). For instance, Itch and its related protein, the neural precursor cell expressing developmentally downregulated (Nedd) 4, have been shown to translocate to detergent-insoluble membrane fractions where they induce ubiquitination and degradation of phospholipase C-γ and protein kinase C-θ (Heissmeyer et al., 2004).

Caspase 3 is an effector member of the caspase family that is also expressed during anergy induction. This protease recognizes proteins with a common DXXD motif, cleaving after the second aspartic residue (Rathmell and Thompson, 1999; Thornberry and Lazebnik, 1998). Caspase 3 is expressed as a proenzyme (32 KDa) that contains a short prodomain, a large (17 KDa) subunit, and a small (12 KDa) subunit. Activation requires proteolytic cleavage between the subunits, where linker segment is typically removed by upstream initiator caspases (Boatright and Salvesen, 2003). T and B cells from *Casp3*$^{-/-}$ mice show hyperproliferative responses, which have been attributed to reduced activation-induced cell death (AICD) (Alam et al., 1999) and to alterations of cell-cycle regulation (Woo et al., 2003), respectively. Caspase 3 also regulates many nonapoptotic cellular processes, such as cell proliferation, cell-cycle regulation, and cell differentiation (Algeciras-Schimnich et al., 2002; Denis et al., 1998). For instance, caspase 3 is implicated in the regulation of the late steps of T cell activation (Alam et al., 1999; Miossec et al., 1997), IL-16 processing (Zhang et al., 1998), dendritic cell maturation (Santambrogio et al., 2005), and erythrocyte (Kolbus et al., 2002) and monocyte (Sordet et al., 2002) differentiation.

In this study we have characterized the activation of caspase 3 in anergic T cells and determined its role in the inhibition of TCR signaling. We show that anergizing stimuli led to increased caspase 3 activation in the absence of apoptotic hallmarks. Activated caspase 3 associated then to the plasma membrane, where it was responsible for the cleavage of GADS and Vav1

after subsequent TCR engagement. Our data identify a role for caspase 3 in nonapoptotic T cells and reveal that caspase 3-dependent proteolytic inactivation of signaling proteins is essential to block TCR signaling in anergic cells and, therefore, to maintain T cell tolerance.

RESULTS

Caspase 3 Is Activated during Anergy Induction in T Cells

We had previously shown that the expression of caspase 3 mRNA was induced in T cells that became unresponsive after being treated with the calcium ionophore ionomycin (Macian et al., 2002). We observed similar results when Th1 cells were anergized with anti-CD3. A clear upregulation of caspase 3 mRNA expression was detected in cells stimulated with anti-CD3 when compared to resting cells or cells stimulated with anti-CD3 and anti-CD28 (Figure 1A). Interestingly, caspase 3 mRNA was still clearly upregulated even when cells received a costimulatory signal in the form of CD28 antibodies after T cells had already been exposed for 2 hr to anti-CD3, which suggests that once the transcriptional complexes that induce caspase 3 transcription are formed, engagement of CD28 might not be sufficient to disrupt them (Figure 1B). Similar results were obtained when DO11.10 Th1 cells were stimulated with CHO cells expressing MHC class II IA^d molecules (CHO-IA^d) loaded with the ovalbumin $Ova_{323-339}$ peptide (OVA), and compared with resting cells or cells activated via CHO-IA^d cells that also expressed B7.2 (CHO-IA^d-B7) (Figure 1C). Stimulated cells also showed some degree of caspase 3 mRNA upregulation, although much smaller than in anergic cells. In order to assess whether caspase 3 proteolytic activity played any role in the induction of T cell anergy, we determined whether increased gene expression correlated with activation. Anergy was induced by coculturing DO11.10 Th1 cells with CHO-IA^d cells loaded with OVA peptide. As controls, we cocultured Th1 cells with unloaded CHO-IA^d cells or with OVA-loaded CHO-IA^d-B7 cells. T cells were then recovered, rested for 3 days, and restimulated with anti-CD3 and anti-CD28. Induction of anergy was confirmed by decreased proliferation and IL-2 production in Th1 cells that had been previously stimulated with OVA-loaded CHO-IA^d, compared to control cells (Figures 1D and 1E). We then determined caspase 3 processing by immunoblot, detected by the presence of a 17 KDa band. Proteolytic activation of caspase 3 was observed only in T cells that had received an anergic stimulus, but not in resting or fully stimulated T cells (Figure 1F). To better characterize the activation of caspase 3, we determined the kinetics of processing during the induction of T cell anergy. Cleavage was evident 6 hr after anergizing stimulation and increased with time (Figure 1G, top). As we had previously shown for caspase 3 expression, activation also seemed to be dependent on calcium signaling, because proteolytic processing was observed with similar kinetics in response to ionomycin (Figure 1G, bottom). In an attempt to identify the mechanism responsible for the activation of caspase 3 in anergic T cells, we determined whether caspase 8 and caspase 9 were also activated in response to anergizing stimuli. We could not detect activation of these proteases in anergic cells (see Figure S1A available online). Similarly, the use of biotin-ZVAD-fmk to pull down activated caspases in anergic cells revealed that only caspase 3, but not caspase 8 or caspase 9, was activated in response to an anergizing stimulus (Figure S1B). Recent reports had indicated that calpain, a calcium-regulated protease, could be involved in the activating cleavage of caspase 3 (McCollum et al., 2002; McGinnis et al., 1999). In order to investigate this possibility in anergic T cells, we anergized Th1 cells with ionomycin in the presence of different concentrations of the calpain inhibitor calpeptin. Calcium-induced caspase 3 cleavage was greatly reduced in the presence of calpeptin, suggesting that calpain may be responsible, at least in part, for the activation of caspase 3 in anergic T cells (Figure 1H). Supporting the important role of calpain in the activation of caspase 3 in anergic cells, ionomycin-induced hyporesponsiveness in Th1 cells was compromised by the inhibition of calpain with calpeptin (Figure S2). Taken together, these results indicated that caspase 3 was activated in T cells in response to calcium signaling induced by anergizing stimuli.

Caspase 3 Activation in Anergic T Cells Does Not Induce Apoptosis

Given the key role that caspase 3 plays during apoptosis, we examined whether its increased activation in anergic T cells could be a consequence of the induction of apoptosis. We analyzed changes in the distribution of membrane phosphatidyl-serine, staining with phycoeritrin (PE)-conjugated Annexin V, and DNA fragmentation, by using a TUNEL assay. Th1 cells were rendered unresponsive by ionomycin treatment and compared with untreated controls, whereas cells treated with staurosporine were used as apoptotic controls. Anergic cells showed a clear increase in the levels of active caspase 3, but no differences in Annexin V or TUNEL staining were observed when compared to control cells (Figures 2A and 2B). Similar results were obtained when T cells were anergized in the presence of CHO-IA^d and OVA and compared with T cells cultured with unloaded CHO-IA^d cells (Figure 2C). To understand the differences in caspase 3 activation between anergic and apoptotic T cells, we measured the magnitude of caspase 3 activity in anergic cells and in apoptotic T cells by using a caspase 3 activity assay (Figure 2D). Results showed that, as suggested by our FACS analysis, the increase in caspase 3 activity in anergic cells was much lower than in apoptotic cells. We could not detect, though, differences in the expression of several negative regulators of caspase activity that might have accounted for the limited caspase 3 activation in anergic cells (Figure S3). We also compared the cleavage of two proapoptotic substrates processed by caspase 3 in apoptotic cells. Although the poly (ADP-ribose) polymerase (PARP) 1 and the inhibitor of caspase-3-activated DNase (ICAD) were clearly cleaved in apoptotic T cells, no processing of these substrates could be detected in anergic cells (Figure 2E).

Thus, activation of caspase 3 in anergic T cells did not result in the proteolytic cleavage of typical proapoptotic caspase substrates and consequently did not cause cell death.

Inhibition of Caspase Activity Compromises Anergy Induction

To determine the role of caspase 3 in the establishment of T cell anergy, we studied the effect of inhibiting its activation during the induction of anergy. For this purpose, we added the caspase

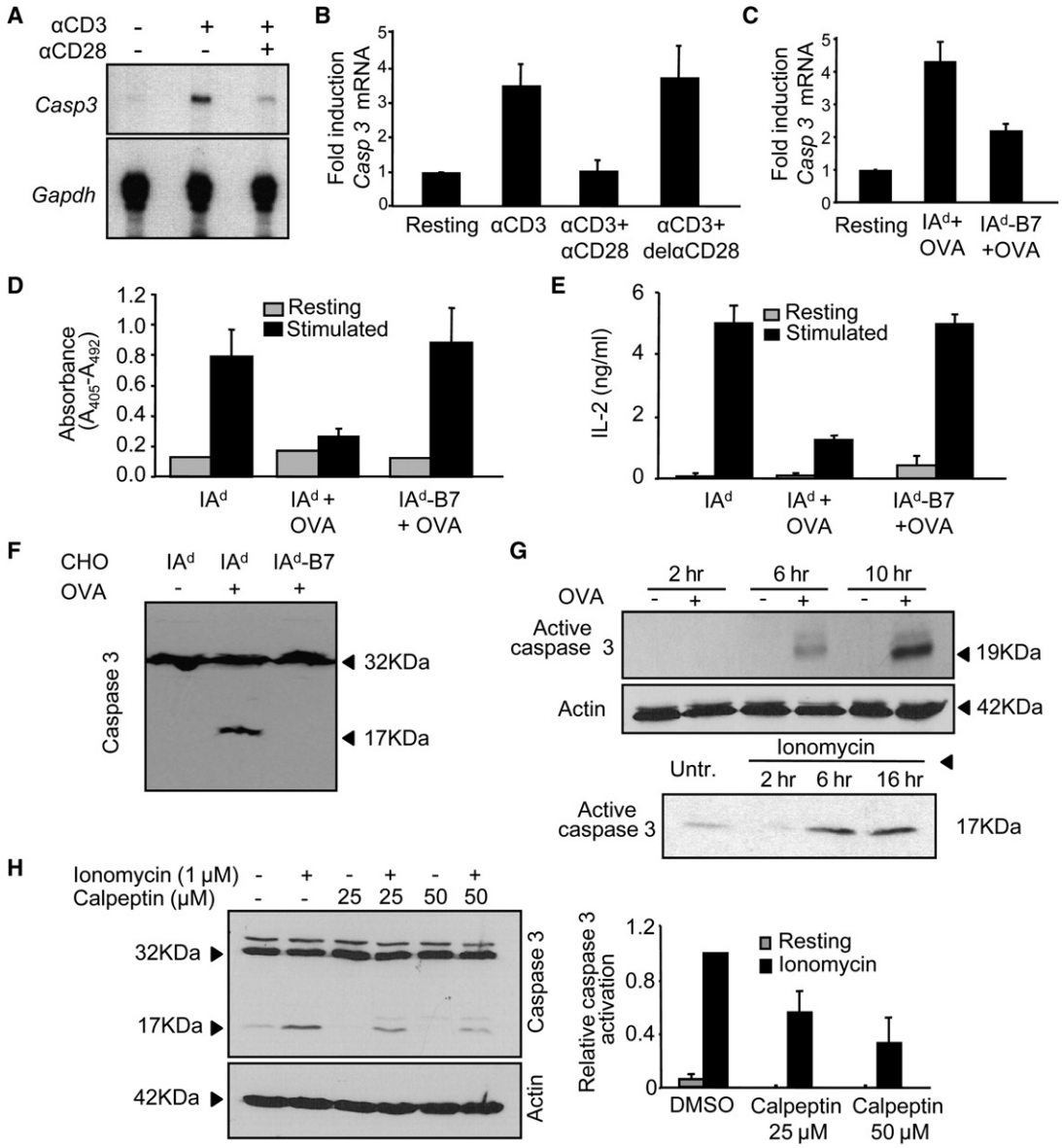

Figure 1. Anergic T Cells Activate Caspase 3

(A and B) Th1 cells were stimulated with anti-CD3 and/or anti-CD28 for 6 hr and caspase 3 mRNA measured by (A) RNase protection assay or (B) qPCR. Where indicated, cells treated with anti-CD3 received anti-CD28 with a 2 hr delay (delαCD28). Results (mean + SEM) are n-fold induction of mRNA expression relative to the control resting cells from at least four experiments.

(C) DO11.10 Th1 cells were cocultured for 6 hr with CHO-IA^d cells loaded or not with OVA or fully stimulated with OVA-loaded CHO-IAd-B7 cells. Amounts of *Casp3* mRNA were analyzed by qPCR. Results are mean + SEM of three experiments.

(D and E) DO11.10 Th1 cells were cocultured for 12 hr with CHO-IA^d cells, loaded or not with OVA or with OVA-loaded CHO-IA^d-B7 cells. After a 3 day resting period, cells were stimulated with anti-CD3+anti-CD28 and (D) BrdU incorporation and (E) IL-2 production measured. Bars show mean + SEM from at least three experiments.

(F) Th1 cells were treated as described in (D) and caspase 3 processing detected by immunoblot.

(G) Th1 cells were stimulated with OVA-loaded CHO-IA^d (top) or ionomycin (bottom). Processing of caspase 3 was measured at different time points with a specific antibody for its active form. Actin was detected as a loading control.

(H) Th1 cells were treated with ionomycin for 6 hr in the presence of calpeptin or control vehicle (DMSO). Caspase 3 activation was detected by immunoblot. Actin was detected as a loading control. Densitometric quantifications of two independent experiments are shown (mean + SEM).

inhibitor Z-VAD to T cells 30 min before being anergized with OVA-loaded CHO-IA^d cells. Inhibition of caspase 3 activation was confirmed in a fraction of the treated cells (Figure 3A). After the anergizing treatment, the caspase inhibitor was washed out and cells were rested for 3 days before being restimulated with anti-CD3 and anti-CD28. We observed a marked impairment in the induction of anergy in Th1 cells anergized in the presence of Z-VAD. Whereas anergic cells show very poor proliferative

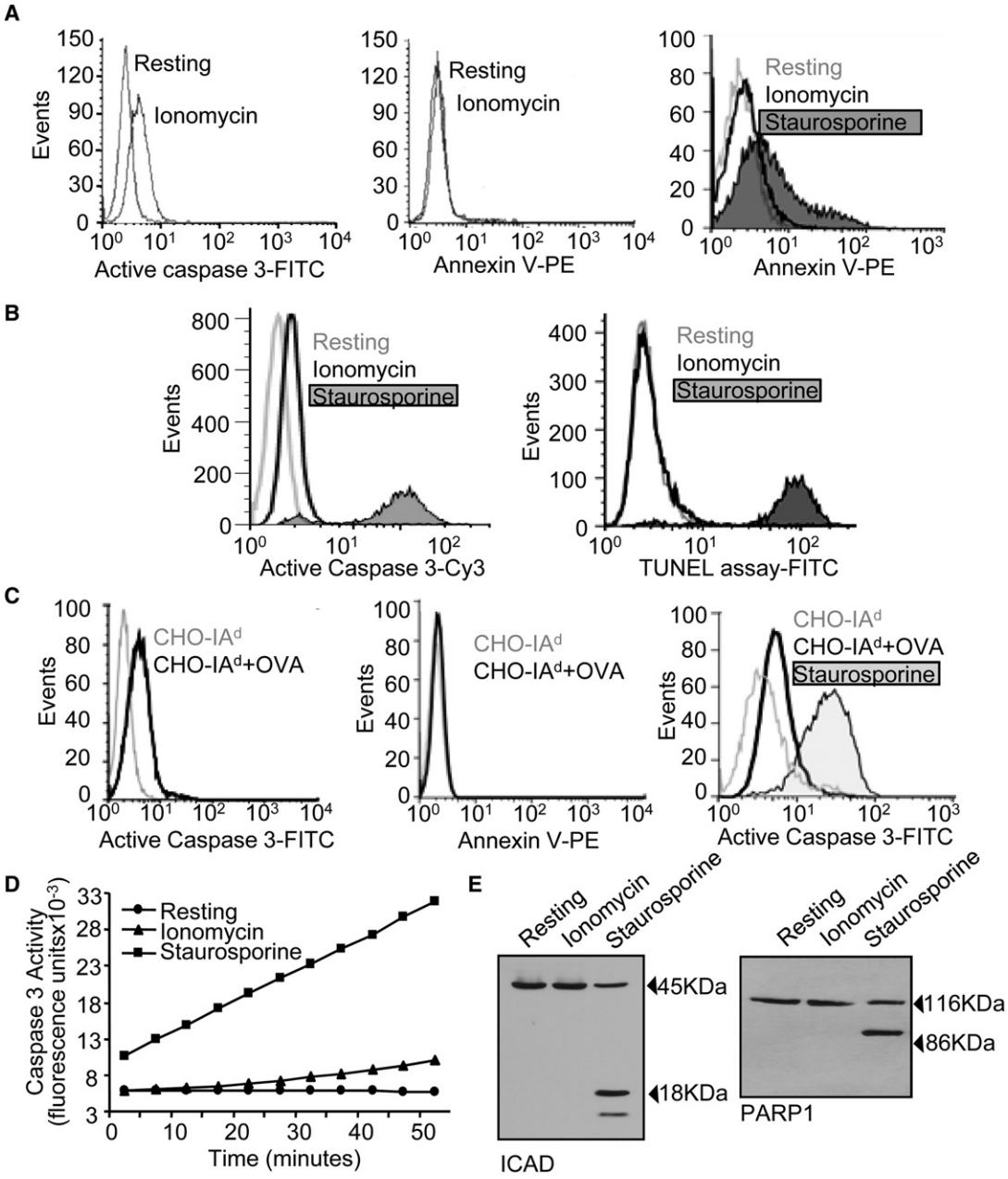

Figure 2. Activation of Caspase 3 Does Not Induce Apoptosis in Anergic T Cells

(A) DO11.10 Th1 cells treated with ionomycin or left resting for 12 hr. T cells were then recovered, washed, stained with an antibody against the active form of caspase 3 (left) or with Annexin V-PE (center), and analyzed by FACS. T cells treated with staurosporine (1 μM, 4 hr) were used as positive control for Annexin V-PE staining (right).

(B) Apoptosis was also detected by TUNEL assay in Th1 cells treated with 1 μM ionomycin for 12 hr or with staurosporine for 4 hr (right). The same cells were also stained for active caspase 3 (left).

(C) Similar analyses were performed in T cells cultured for 12 hr with CHO-IA^d cells loaded or not with OVA.

(D) Caspase 3 activity was measured with a fluorogenic caspase-3 substrate in cell lysates from Th1 cells left resting, anergized with ionomycin for 12 hr, or treated with 1 μM staurosporine for 4 hr.

(E) Th1 cells were left resting, incubated with 1 μM ionomycin for 12 hr, or treated with 1 μM staurosporine for 4 hr. Total cell lysates were analyzed by immunoblot with antibodies that recognized the proapoptotic caspase 3 substrates PARP1 and ICAD.

responses to stimulation, cells anergized in the presence of Z-VAD proliferated upon restimulation almost as well as untreated or previously stimulated control T cells (Figure 3B). To confirm these results, cells were also anergized with irradiated splenocytes loaded with OVA and a cytotoxic T-lymphocyte antigen 4 (CTLA-4)-Ig fusion protein to block B7 molecules. With this

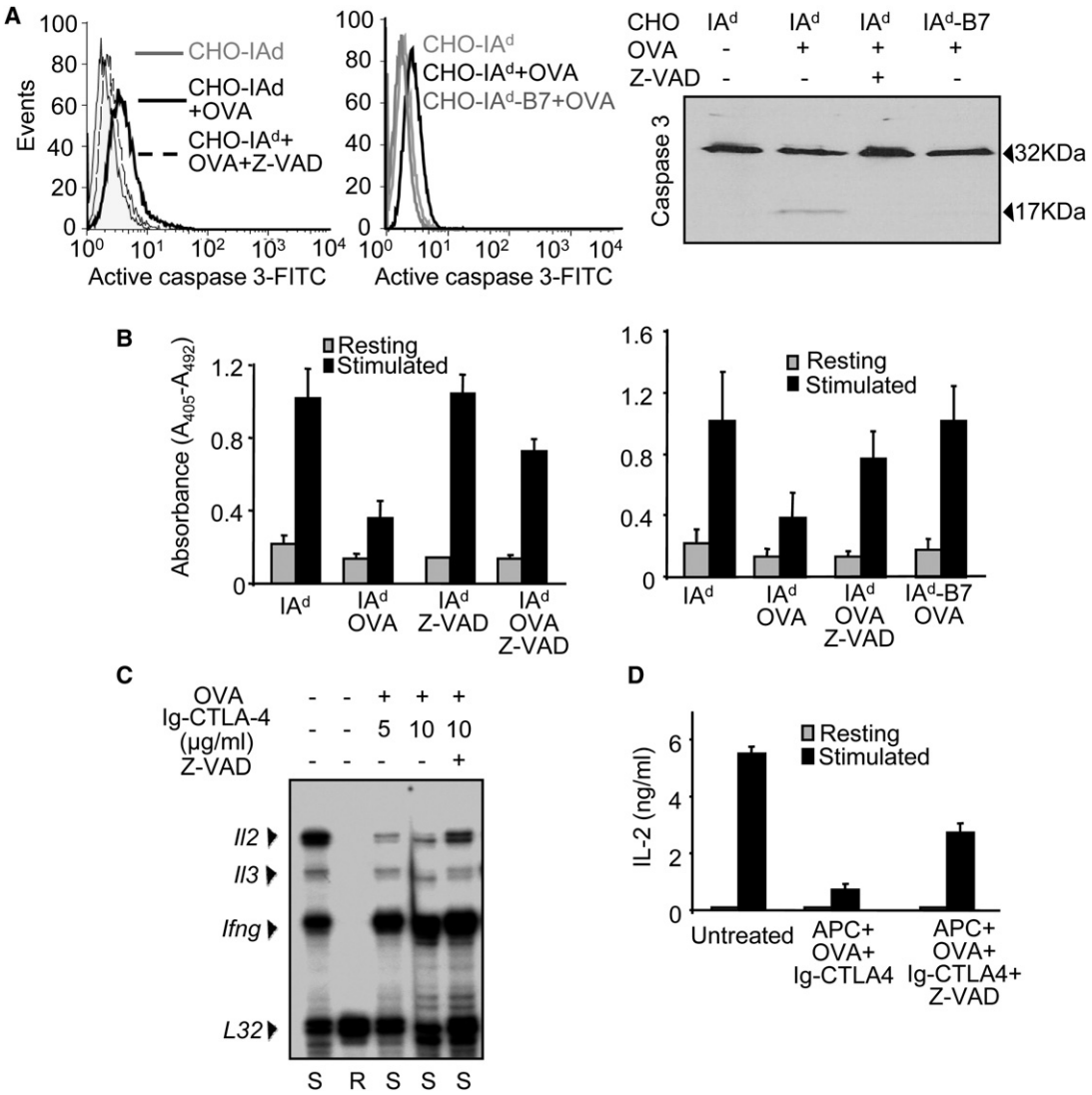

Figure 3. Inhibition of Caspase Activity Compromises Anergy Induction in T Cells

(A and B) DO11.10 Th1 cells were anergized by coculture for 12 hr with OVA-loaded CHO-IA^d cells. Z-VAD or control vehicle (DMSO) were added where indicated. Th1 cells cocultured with CHO-IA^d cells or with OVA-loaded CHO-IA^d-B7 cells were used as controls.

(A) Z-VAD inhibition of caspase 3 activation was determined by intracellular staining for active caspase 3 or immunoblot.

(B) Degree of anergy after treatments was determined by measuring BrdU incorporation after anti-CD3+anti-CD28 stimulation. Values are mean + SEM of at least three different experiments.

(C and D) DO11.10 Th1 cells were anergized with irradiated splenocytes preincubated with OVA and CTLA-4-Ig. Z-VAD was added where indicated. After 12 hr, T cells were recovered, washed, and rested for 3 days before restimulation with anti-CD3+anti-CD28. IL-2 production was measured by (C) RNase protection assay or (D) ELISA. Untreated control cells were also analyzed. S, stimulated; R, resting. Values are mean + SEM of at least three different experiments.

protocol, cells anergized in the presence of Z-VAD produced, after restimulation, several times more IL-2 than cells anergized without Z-VAD (Figures 3C and 3D). Our data suggested, therefore, that caspase activity was required for the induction of anergy in T cells in response to partial stimulation.

Caspase 3 Is Necessary to Induce Anergy in T Cells

We next addressed whether specifically caspase 3 activity was required to induce T cell hyporesponsiveness. Th1 cells were transfected with a plasmid expressing a shRNA specific for caspase 3 mRNA, which led to a marked reduction of caspase 3 expression (~80%) and almost no detectable caspase 3 activation after anergy induction (Figure 4A and data not shown). Inhibition of IL-2 expression after anergizing treatment with ionomycin was significantly impaired in cells expressing a specific shRNA for *Casp3* compared to control cells (IL-2 expression reduced to 56% and 16%, respectively, after anergizing treatment; $p < 0.01$) (Figure 4B). We confirmed these results by using Th1 cells from *Casp3*$^{-/-}$ mice. Cells were anergized with ionomycin for 12 hr and IL-2 production was measured after restimulation with anti-CD3 and anti-CD28. As previously seen in cells in which *Casp3* expression had been inhibited with a shRNA, *Casp3*$^{-/-}$

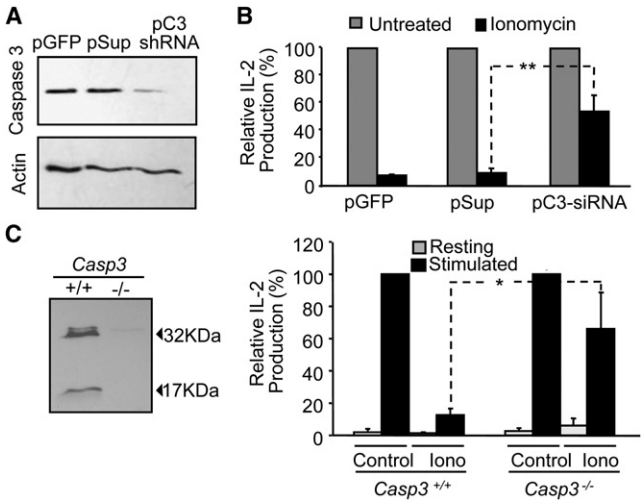

Figure 4. Blocking Caspase 3 Expression Interferes with the Induction of Anergy

(A) Th1 cells were transfected with a GFP-expressing vector only or together with the pC3-shRNA, which expresses a specific caspase 3 shRNA or control pSup vector. Cells were then sorted for GFP expression and treated with 1 μM ionomycin for 12 hr, and caspase 3 and actin were detected by immunoblot.

(B) Untreated and ionomycin-treated control T cells and T cells transfected with a plasmid expressing a *Casp3* shRNA were stimulated with anti-CD3+anti-CD28 and IL-2 production measured by ELISA. Bars show percentage of IL-2 expression relative to control stimulated cells and are mean + SEM of three experiments. $^{**}p < 0.01$.

(C) $CD4^+$ T cells were isolated from $Casp3^{-/-}$ mice and wild-type littermates. Absence of caspase 3 expression in $Casp3^{-/-}$ cells was confirmed by immunoblot. T cells were differentiated under Th1 skewing conditions and then left resting or treated with 1 μM ionomycin for 12 hr. Cells were then washed and left resting for 6–8 hr before restimulation with anti-CD3+anti-CD28. Inhibition of IL-2 production in $Casp3^{-/-}$ T cells was compared with that of wild-type anergized T cells. Bars show percentage of IL-2 expression in anergic cells compared with control stimulated cells and are mean + SEM of six mice. $^{*}p < 0.05$.

T cells were significantly less susceptible to become anergic than were $Casp3^{+/+}$ T cells (IL-2 expression reduced to 67% and 13%, respectively, after treatment with ionomycin; $p < 0.05$) (Figure 4C). Under these conditions, we could not detect differences in the degree of apoptosis induced in anergic wild-type or $Casp3^{-/-}$ T cells after stimulation with anti-CD3 and anti-CD28 (Figure S4). These data demonstrated, thus, that calcium-induced caspase 3 activity was necessary for the establishment of T cell unresponsiveness.

Caspase 3 Cleaves GADS and Vav1 in Anergic T Cells

Several TCR signaling components have been reported as caspase substrates during apoptosis or after CD95-induced inhibition of antigen receptor signaling, including the Grb2-related adaptor downstream of shc (GADS) (Berry et al., 2001), the guanine-nucleotide exchange factor Vav1 (Hofmann et al., 2000), the TCRζ chain (Gastman et al., 1999), and PLCγ (Bae et al., 2000). By analyzing total cell extracts from anergic T cells, we were able to detect degradation of GADS (Figure 5A). No detectable cleavage was found in other substrates (data not shown). This cleavage was caspase 3 dependent as indicated by the fact that it did not occur in $Casp3^{-/-}$ Th1 cells (Figure 5B). Similar results were obtained when T cells from DO11.10 mice were tolerized in vivo by oral feeding with ovalbumin (Figure 5C, left). Orally tolerized $CD4^+$ T cells activated caspase 3 and showed cleavage of GADS and Vav1 (Figure 5C, right).

Amounts of GADS and Vav1 cleavage detected were moderate, suggesting that only a reduced fraction of the total cell content of those proteins was being processed by caspase 3. One possible explanation could be that only the fraction of GADS and Vav1 recruited to the TCR-associated signaling complex could become target of caspase 3 activity. To test this hypothesis, we first analyzed the subcellular localization of active caspase 3 in anergic T cells. The active form of caspase 3 was detected only in the pellet but not in the soluble fraction of a S100 lysate, suggesting membrane localization (Figure 6A, left). Further subfractionation assays showed that active caspase 3 appeared clearly associated with the plasma membrane fraction, but not with those containing other organelles or with nuclear fractions (Figure 6A, right). Purity of the fractions was assessed with antibodies against connexin43, GM130, and Bcl-2 (data not shown). This specific subcellular localization would provide a way to restrict caspase 3 activity to signaling molecules recruited to the membrane during T cell activation. In order to test this hypothesis, we analyzed the cleavage of GADS and Vav1 in anergic cells after restimulation. Th1 cells derived from $Casp3^{+/+}$ or $Casp3^{-/-}$ mice were treated with ionomycin in the presence or absence of the caspase inhibitor Z-VAD for 12 hr to induce anergy. Cells were then washed and left resting for several hours before restimulation. No differences in the levels of Annexin V staining could be detected between stimulated anergic and nonanergic cells, ruling out the possibility that increased caspase 3-mediated processing might result from increased apoptosis in anergic T cells (Figure 6B). As hypothesized, stimulation of T cells led to increased GADS and Vav1 cleavage in anergic cells (Figures 6C and 6D). A very low degree of processing could also be observed in nonanergic cells, which could result from a fraction of cells that might have been only suboptimally stimulated in our assay, or from some small amount of apoptosis induced during stimulation. As shown before, this cleavage was caspase 3 dependent, as indicated by the fact that it was blocked by a caspase inhibitor (Figure 6C) and was absent in $Casp3^{-/-}$ T cells (Figure 6D).

If the blockade of TCR signaling was a consequence of the caspase-dependent cleavage of Vav1 and GADS, we should be able to reverse this effect by using caspase-resistant forms of these proteins. Th1 cells were transfected with plasmids expressing either wild-type or caspase-resistant forms of GADS and Vav1 bearing mutations in the caspase-recognition site that rendered them resistant to caspase 3-mediated cleavage (Berry et al., 2001; Hofmann et al., 2000). Ionomycin-induced hyporesponsiveness, measured as decreased IL-2 production upon restimulation, was similar in control cells and in cells transfected with wild-type proteins, but was significantly reduced in cells expressing the caspase-resistant GADS or Vav1. This effect was even more pronounced when both proteins were coexpressed together (Figure 6E).

To exclude the possibility that the cleaved fragments might have an effect on T cell activation, we transduced primary T cells with retrovirus expressing the different fragments that

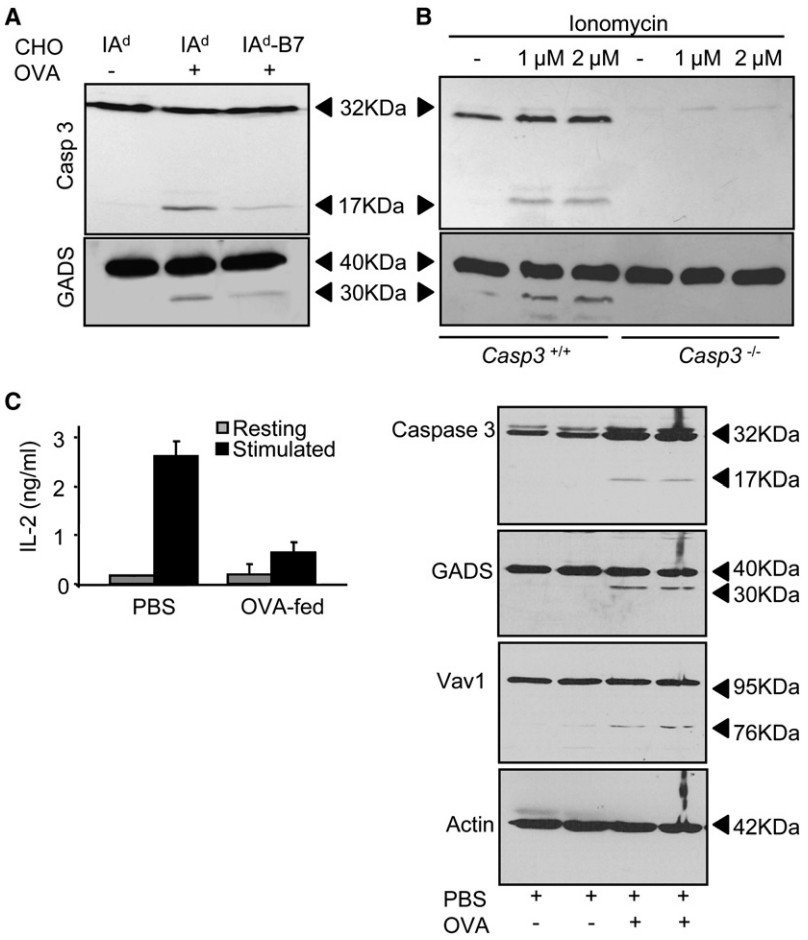

Figure 5. Caspase 3 Cleaves GADS and Vav1 in Anergic T Cells

(A) DO11.10 Th1 cells were cocultured for 12 hr with CHO-IA^d cells loaded or not with OVA, or with OVA-loaded CHO-IA^d-B7 cells. T cells were then recovered and caspase 3 and GADS processing determined.

(B) *Casp3*$^{-/-}$ and wild-type littermate Th1 cells were treated for 12 hr with ionomycin and processing of caspase 3 and GADS detected by immunoblot.

(C) DO11.10 mice were orally tolerized with ovalbumin. Seven days later, CD4$^+$ T cells were isolated and stimulated with anti-CD3+anti-CD28. IL-2 production was measured by ELISA. Proteolytic cleavage of caspase 3, GADS, and Vav1 was determined by immunoblot. Results are from two different mice from each group (mean + SEM).

would result from the caspase 3-mediated cleavage of Vav1 or GADS. Cells expressing either of the two different fragments or both fragments at the same time were then stimulated with anti-CD3 and anti-CD28. The expression of those fragments did not cause any effect on IL-2 production, suggesting that the loss of function seen in anergic T cells was likely a consequence of the cleavage of these proteins and not of alterations in signaling pathways caused by the cleaved fragments (Figure 6F).

Collectively, these results indicated that active caspase 3 was recruited to the plasma membrane in anergic cells where it could inhibit T cell activation by cleaving and inactivating GADS and Vav1.

Caspase 3 Cleavage of Vav1 Prevents Its Recruitment to the Plasma Membrane

Vav1 is recruited to the membrane upon engagement of the TCR. To determine whether cleavage by caspase 3 could prevent this recruitment, we analyzed Vav1 subcellular localization after TCR engagement in control and anergic T cells. As expected, in resting cells Vav1 resided mostly in the cytosol, but was readily recruited to the membrane upon stimulation. This effect was blocked in anergic cells, which showed markedly reduced Vav1 recruitment to the plasma membrane after stimulation with anti-CD3 and anti-CD28 (Figure 7A). To corroborate the effect that cleavage of Vav1 had on its subcellular localization, we analyzed membrane recruitment in anergized cells previously transduced with retroviruses that expressed either wild-type or a caspase 3-resistant Vav1. In control cells transduced with a virus expressing GFP, recruitment of Vav1 to the plasma membrane upon activation was greatly reduced in anergic T cells. Overexpression of a wild-type Vav1 resulted in a small increase in membrane recruitment that was not significant and likely due to the overexpression of this protein. However, the expression of the caspase 3-resistant Vav1 completely restored recruitment of Vav1 to the membrane in anergic cells to a degree similar to that found in nonanergic control T cells (Figure 7B). These data further supported that caspase 3 localization to the plasma membrane in anergic T cells resulted in the inhibition TCR signaling by allowing this protease to target and cleave Vav1 and prevent its proper activation-induced recruitment to the membrane.

DISCUSSION

It is becoming increasingly evident that caspases are versatile proteases that control many cellular functions other than apoptosis. Here we show that caspase 3 negatively regulates TCR signaling in anergic T cells. Anergy is induced in T cells by suboptimal stimulation that causes sustained calcium signaling and the consequent activation of the calcineurin-NFAT axis (Bandyopadhyay et al., 2007; Borde et al., 2006; Heissmeyer et al., 2005). We have shown that under these conditions, the expression of a specific set of genes is upregulated (Macian et al., 2002), which encode proteins responsible for the inhibition of T cell activation (Anandasabapathy et al., 2003; Heissmeyer et al., 2004; Jeon et al., 2004; Olenchock et al., 2006; Su et al., 2006; Zha et al., 2006). Caspase 3 is also expressed in anergic T cells in a calcium/NFAT-dependent manner (Macian et al., 2002; Kaji et al., 2003). Our results show now that caspase 3 is activated in nonapoptotic anergic T cells and that the activity of this protease is necessary to maintain anergic T cells hyporesponsive.

Originally described as a crucial regulator of apoptosis, caspase 3 has also been shown to regulate other processes

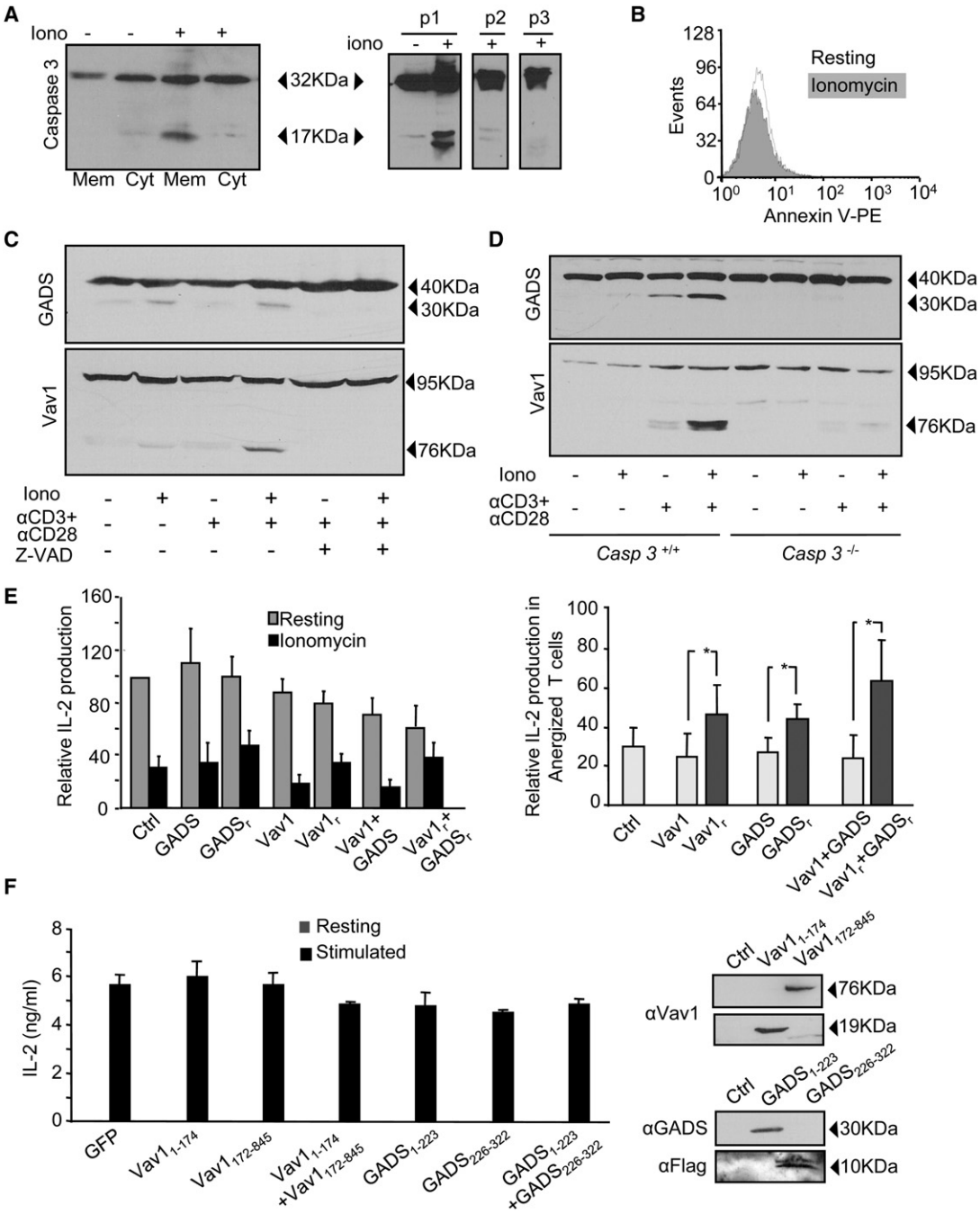

Figure 6. Active Caspase 3 Associates to the Plasma Membrane in Anergic T Cells Where It Cleaves GADS and Vav1 after TCR Stimulation

(A) S100 fractions were prepared from resting Th1 cells or cells treated with 1 μM ionomycin for 12 hr (left). Lysates from those cells were also fractionated by differential centrifugation in sucrose gradients into fractions containing the plasma membrane (P1), mitochondria, lysosomes, endosomes, and peroxisomes (P2) and the Golgi and ER (P3) (right). The presence of active caspase 3 in all fractions was detected by immunoblot.

(B and C) Th1 cells from *Casp3*$^{-/-}$ or wild-type mice were treated with 1 μM ionomycin for 12 hr, washed, and rested for 4 hr before stimulation with anti-CD3+anti-CD28 for 6 hr. Annexin V staining was detected by FACS in stimulated cells (B) and proteolytic processing of GADS and Vav1 determined in lysates from these cells (C).

(D) Th1 cells were left untreated or treated with 1 μM ionomycin for 16 hr, washed, and left resting for 4 hr before restimulation, in the presence or absence of Z-VAD. After 6 hr, cells were collected and proteolytic cleavage of GADS and Vav1 detected by immunoblot.

(E) Th1 cells from C57BL6/J mice were transfected with plasmids expressing wild-type or caspase-resistant (GADS$_r$ and Vav1$_r$) murine GADS and/or Vav1. Amounts of DNA used were adjusted to obtain similar levels of expression of all proteins. Control cells were transfected with pGFP. 36 hr after transfection, cells were rested or treated with ionomycin 1 μM for 12 hr. IL-2 was measured by ELISA after stimulation with anti-CD3+anti-CD28. Left panel shows normalized (to control untreated cells) IL-2 values, whereas right panel shows percentage of IL-2 expression in anergic cells compared with control cells in each experimental condition. Bars are mean + SEM of four experiments. *$p < 0.05$.

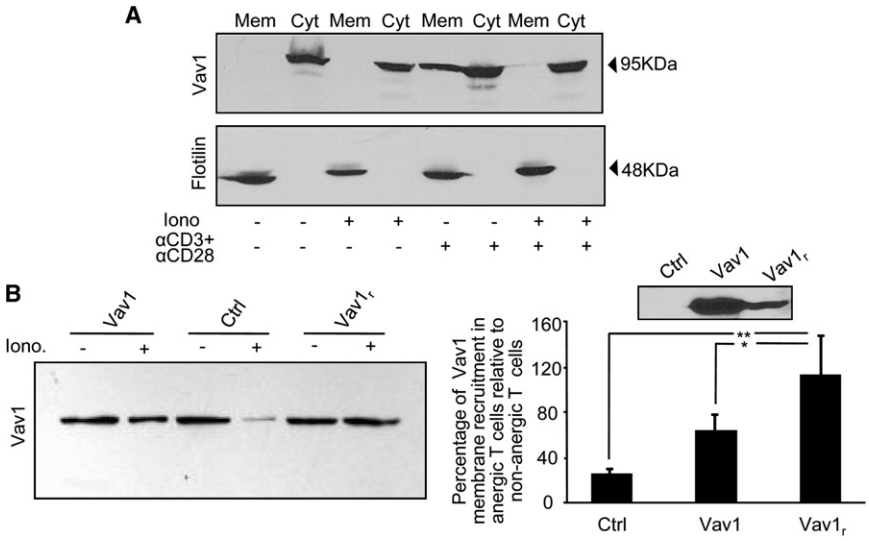

Figure 7. Caspase 3-Mediated Cleavage Prevents Activation-Dependent Recruitment of Vav1 to the Membrane in Anergic T Cells

(A) Vav1 membrane recruitment was determined by immunoblot of pellet (mem) and supernatant (cyt) S100 fractions of control and anergic Th1 cells stimulated with anti-CD3+anti-CD28. Detection of flotillin1 was used as a membrane marker.

(B) Th1 cells, transduced with virus expressing wild-type or caspase-resistant ($Vav1_r$) Vav1, were sorted, and the translocation of Vav1 to the membrane in control and anergized cells was determined after stimulation with anti-CD3+anti-CD28. Densitometric quantification of six experiments (mean + SEM) is shown. $^{**}p < 0.01$, $^{*}p < 0.05$.

including skeletal muscle differentiation and the maturation of erythroid precursors, monocytes, and dendritic cells (De Maria et al., 1999; Fernando et al., 2002; Santambrogio et al., 2005; Sordet et al., 2002; Wong et al., 2004). In B cells, caspase 3 regulates entry into the cell cycle by cleaving p21 and preventing the formation of active cyclin/CDK complexes (Woo et al., 2003). In T cells, caspase 8 is activated upon stimulation and controls T cell proliferation (Alam et al., 1999; Kennedy et al., 1999). Blocking caspase activity during T cell stimulation leads to decreased activation-induced proliferation and cytokine production, which is also evident in caspase 8-deficient T cells (Alam et al., 1999; Falk et al., 2004; Kennedy et al., 1999; Salmena and Hakem, 2005; Salmena et al., 2003). The mechanisms responsible for this effect may reside in the ability of active caspase 8 to induce NF-κB activation and to regulate cell cycle in T cells (Falk et al., 2004; Su et al., 2005). Here we report that another member of the caspase family of proteases, caspase 3, has a different role in T cell physiology and negatively regulates TCR signaling in anergic T cells. Although we cannot rule out that other mechanisms may also contribute to the activation of caspase 3 in response to tolerogenic stimuli, our results indicate that calcium signaling induces the calpain-mediated activation of this protease in anergic T cells. Caspase 3 activation has also been reported in fully stimulated T cells, but with delayed kinetics compared to its activation in anergic cells (Alam et al., 1999; Misra et al., 2005). It is possible, therefore, that caspase 3 may contribute to terminate T cell activation through a mechanism similar to the one that operates in anergic T cells.

Ubiquitin ligases, such as Itch and Cbl-b, have been implicated in downregulating TCR signaling by targeting components of the TCR signalosome to the endocytic pathway, leading to their functional inactivation or degradation (Heissmeyer et al., 2004). We propose that caspase 3-mediated cleavage of proteins involved in TCR signal transduction, such as GADS and Vav1, is also responsible for maintaining a status of hyporesponsiveness in anergic T cells. GADS is an adaptor protein that interacts with the Src homology 2-domain-containing leukocyte phosphoprotein of 76 KDa (SLP-76) and the linker for activation of T cells (LAT) (Boerth et al., 2000; Yankee et al., 2004). GADS-deficient T cells have profound defects in TCR signaling that severely affect thymocyte development (Yankee et al., 2004). Induction of apoptosis or CD95 engagement leads to the caspase-dependent cleavage of GADS, which blocks productive TCR signaling (Berry et al., 2001; Yankee et al., 2001). Vav1, a nucleotide exchange factor for the RhoA, Rac1, and cdc42 GTPases (Fujikawa et al., 2003; Tybulewicz et al., 2003), is also cleaved by caspase 3 in apoptotic T cells (Hofmann et al., 2000). The resulting fragment fails to induce p38 activation and cannot support transcription from NFAT, AP-1, or NF-κB reporter plasmids (Hofmann et al., 2000). We report here that cleavage of these two proteins occurs also in anergic T cells. Whereas in apoptotic cells inhibition of these signaling pathways may prevent aberrant cytokine synthesis in a cell destined to die, in anergic T cells this same mechanism would engage to block T cell activation. We have confirmed that the cleavage of GADS and Vav1 in anergic T cells is mediated by caspase 3. Blocking caspase 3 expression with siRNA or using T cells from caspase 3-deficient mice leads to defective anergy induction, which correlates with lack of GADS and Vav1 cleavage. Furthermore, cells that express caspase-resistant forms of these proteins are also resistant to anergy induction. Nevertheless, some degree of hyporesponsiveness is still induced in these cells, which might be due to the activation of other mechanisms that also dampen TCR signaling in anergic T cells. Although the results from the experiments performed with caspase-resistant Vav1 and GADS strongly suggest a critical role for these two proteins in controlling TCR signaling in anergic T cells, we cannot rule out that other targets of caspase 3 exist in anergic T cells and that their proteolytic processing also contributes to keep anergic cells hyporesponsive.

(F) Th1 cells were transduced with retrovirus expressing the N-terminal ($Vav1_{1-174}$, $GADS_{1-223}$) and/or the C-terminal ($Vav1_{172-845}$, $GADS_{226-322}$) fragments of Vav1 and GADS. Expression of these peptides was verified by immunoblot in infected NIH 3T3 cells. IL-2 production was measured in sorted T cells after stimulation with anti-CD3+anti-CD28. Bars are mean + SEM of at least two experiments.

The role of caspase 3 as a tolerance-inducing factor does not seem to be restricted to the induction of clonal anergy in Th1 cells, because we can also detect caspase 3 activation and cleavage of GADS and Vav1 in T cells from orally tolerized mice. These findings support that activation of the calcium-NFAT-dependent expression of caspase 3, and likely several other genes, may constitute a basic mechanism for the induction of T cell tolerance.

Substrate specificity may underlie the different roles of caspase 3 in different tissues and physiological conditions and explain why its activation does not cause apoptosis in anergic T cells. It is still not clear how this specificity might be achieved. We could not detect differences in the expression of several negative regulators of caspase activation; however, caspase 3 activity in anergic cells was much lower than that detected in apoptotic cells. More importantly, active caspase 3 seemed to be confined to the plasma membrane, where accessibility to substrates would be restricted. Supporting this idea, we could not detect significant changes in the levels of total Vav1 in anergic T cells; however, the presence of this protein in the plasma membrane was drastically reduced in anergic cells, which could be restored by expressing a caspase-resistant form of Vav1. Hence, both quantitative and qualitative differences in caspase 3 activity between anergic and apoptotic T cells may explain the different outcomes of the activation of this protease.

Maintenance of T cell tolerance may represent just a part of a global role of caspases as immunomodulators. Apart from its involvement in AICD and anergy, caspase 3 has also been shown to negatively regulate B cell progression into the cell cycle (Woo et al., 2003). Furthermore, basal caspase 3 activity is required to maintain dendritic cells in an immature state, and its inhibition by activating stimuli induces dendritic cell maturation and productive presentation of antigens (Santambrogio et al., 2005; Wong et al., 2004).

Caspase 3 plays, thus, a crucial role in the establishment and maintenance of T cell tolerance. Calcium signals engaged in response to tolerogenic stimuli are responsible for the activation of caspase 3, which inhibits T cell activation by inactivating key proteins involved in transducing signals from the TCR.

EXPERIMENTAL PROCEDURES

Mice

Mice were maintained in pathogen-free conditions. All animal work was performed according to the guidelines set by the Institutional Animal Care Committee of the Albert Einstein College of Medicine. 4- to 6-week-old C57BL6/J, BALB/c, and DO11.10 mice were purchased from Jackson Laboratories. *Casp3*$^{-/-}$ mice (Woo et al., 2003) were kindly provided by T.W. Mak.

Cell Culture

Primary CD4$^+$ T cells were isolated from lymph nodes and spleens via magnetic beads (Invitrogen), stimulated with 0.5 μg/ml plate-bound anti-CD3 and 0.5 μg/ml anti-CD28 (BD), and differentiated for 7 days with 10 ng/ml IL-12 (Cell Science), 10 μg/ml anti-IL-4, and 10 U/ml of recombinant human IL-2. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, nonessential amino acids, essential vitamins (Cambrex), and 50 μM 2-mercaptoethanol. Chinese hamster ovary (CHO) cells expressing IA^d with or without B7.2 were kindly provided by S. Bhatia.

Induction of T Cell Tolerance

Four different systems were used to induce T cell tolerance. (1) Mitomycin C (Sigma)-pretreated CHO-IA^d cells loaded with 10 μM OVA peptide were cocultured with DO11.10-derived Th1 cells for 12 hr. Th1 cells were recovered and rested for 3 days before being analyzed. (2) Th1 cells were treated with 1 μM ionomycin (EMD) for 12 hr, washed, and rested for 4 hr before being restimulated. (3) DO11.10 Th1 cells were cultured for 12 hr with irradiated BALB/c mice splenocytes preincubated with 10 μM OVA and 10 μg/ml CTLA-4-Ig (Chimerigen). Th1 cells were recovered, washed, and rested for 3 days before being analyzed. In all procedures, the caspase inhibitor Z-VAD (EMD) was added at a final concentration of 100 μM where indicated during anergy induction and then washed out. (4) Oral tolerance was induced by the administration of a single intragastric dose of 25 mg of ovalbumin (Sigma) to DO11.10 mice. On day 7, CD4$^+$ T cells were isolated and analyzed.

ELISA

2.5 or 5 × 10^4 Th1 cells were stimulated in 96-well plates. Supernatants were collected 24 hr after stimulation and IL-2 levels were measured in a sandwich ELISA (BD).

Proliferation Assay

25 × 10^3 Th1 cells were stimulated in 96-well plates. 60 hr later, 5-Bromo-2′-deoxy-uridine (BrdU) was added for 12 hr. Incorporation of BrdU was measured by ELISA according to manufacturer's instructions (Roche).

Immunoblotting

Whole-cell extracts were prepared by boiling cell pellets directly in SDS to prevent proteolysis during cell lysis. Antibodies against caspase 3 and 9 and cleaved caspase 3 (Cell Signaling), GADS (Upstate Biotech), Vav1 (Santa Cruz Biotech), FLIP (EMD), and Caspase 8, XIAP, Flotillin1, Connexin-43, GM130, and Bcl-2 (BD) were used. Where indicated, the calpain inhibitor calpeptin (EMD) or the caspase inhibitor Z-VAD were added to cells 30 min before adding 1 μM ionomycin for 6 to 12 hr. Densitometric quantification of the immunoblotted membranes and stained gels was done with ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij).

Detection of Active Caspases

Total cell extracts were prepared by lysing 5 × 10^6 cells in 1 ml of RIPA buffer with a cocktail of protease inhibitor (Roche). 100 μM of biotin-VAD-fmk (EMD) were added for 4 hr at 4°C, followed by 50 μl of streptavidin agarose beads (Invitrogen) and incubated for 4 hr at 4°C. Agarose beads were washed three times with RIPA buffer and boiled directly in SDS.

Apoptosis Detection

Apoptosis was determined with Annexin V-PE apoptosis detection kit (BD) or by TUNEL assay with the In situ Cell Death Detection kit (Roche). Stained cells were analyzed on a FACSCAN (Beckton-Dickinson).

Intracellular Staining

To detect caspase 3 activation, T cells were fixed in 4% paraformaldehyde, permeabilized in PBS/1% BSA/0.5% saponin, and incubated with an antibody against the active fragment of caspase-3. A secondary FITC or Cy3-conjugated antibody was used. Cells were analyzed on a FACSCAN.

Caspase 3 Activity

T cells were plated in 96-well pates at 5 × 10^4 cells/well. Fluorogenic caspase 3 substrate IX (EMD) was added at a final concentration of 50 μM. Fluorescence was measured with a plate reader fluorimeter (BMG) every 5 min for 1 hr at 37°C.

RNase Protection Assay

Total RNA was obtained from T cells with Trizol Reagent (Invitrogen) and analyzed with the RiboQuant multiprobe RNase protection kit with the mCK-1b or the mAPO-1 probes (BD).

Quantitative RT-PCR

Total RNA was prepared from T cells with Trizol Reagent and used to synthesized cDNA via a Superscript kit (Invitrogen). qPCR was performed with

a SYBR green master mix (Stratagene) as described before (Macian et al., 2002) with specific primers to amplify a fragment from the caspase 3 cDNA (5′-ACGCGCACAAGCTAGAATTT, 5′-CTTTGCGTGGAAAGTGGAGT).

Plasmids and T Cell Transfection

Plasmids expressing the wild-type murine GADS and a caspase resistant mutant form (D235A) were kindly provided by C.J. McGlade (Berry et al., 2001; Liu and McGlade, 1998). A wild-type Vav-1-expressing construct was kindly provided by X.R. Bustelo. Vav1 protein resistant to caspase cleavage (D150A/D161A) was generated by PCR-based site-directed mutagenesis. 5 to 20 μg of DNA were introduced in T cells with a nucleofector device (AMAXA) according to the manufacturer's protocol. Cells were anergized 36 hr after transfection.

Retroviral Infection of Primary Th1 Cells

GADS, $GADS_{1-223}$, flag-$GADS_{226-322}$, Vav1, $Vav1_{1-174}$, and $Vav1_{172-845}$ cDNAs were subcloned in the RV-IRES-GFP vector (Macian et al., 2002). Phoenix ecotropic packaging cells were transfected with a calcium/phosphate method with the different retroviral vectors. 48 hr after transfection, retroviral supernatants were collected, supplemented with polybrene (8 μg/ml), and used to infect T cells 24 hr after stimulation. Infected cells were sorted for GFP expression.

RNA Interference

A murine caspase 3-specific shRNA (5′-GTAAAGACCATACATGGGA-3′) was introduced into the pSUPER (Oligoengine). 20 μg of DNA (1:4 ratio pMaxGFP and pC3RNAi) were transfected into 5×10^6 T cells. GFP-positive cells were sorted 36 hr after transfection and analyzed.

Subcellular Fractionation

Cells were resuspended in ice-cold fractionation buffer (10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT with protease inhibitors) and disrupted by sonication. Nuclei were pelleted by centrifugation (700 × g, 10 min, 4°C) and supernatants centrifuged at 100,000 × g for 1 hr at 4°C to obtain the membrane (P100) and the cytosolic (S100) fractions. Alternatively, cells were resuspended in ice-cold 0.25 M sucrose and disrupted by sonication. Nuclei were pelleted and supernatants centrifuged at 1450 × g for 10 min at 4°C to obtain the membrane fraction (P1). Centrifugation at 17,000 × g, 10 min, at 4°C was performed to obtain the organelles fraction (mitochondria, lysosomes, endosomes, and peroxisomes) (P2). A final centrifugation at 100,000 × g (1 hr at 4°C) was performed to separate endoplasmic reticulum and Golgi (P3).

Statistical Analysis

Differences in the inhibition of IL-2 production in anergic T cells were analyzed with a Student's t test (paired, two-tails).

SUPPLEMENTAL DATA

Supplemental Data include four figures and can be found with this article online at http://www.immunity.com/cgi/content/full/29/2/193/DC1/.

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