γ-Associated Protein of 70 kDa (ZAP-70), but Not Syk, Tyrosine Kinase Can Mediate Apoptosis of T Cells through the Fas/Fas Ligand, Caspase-8 and Caspase-3 Pathways

Lingwen Zhong, Chun-Hua Wu, Wen-Hui Lee and Chih-Pin Liu

_J Immunol_ 2004; 172:1472-1482; doi: 10.4049/jimmunol.172.3.1472
http://www.jimmunol.org/content/172/3/1472

---

Why _The JI_?

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

---

**References** This article cites _68 articles_, 33 of which you can access for free at:
http://www.jimmunol.org/content/172/3/1472.full#ref-list-1

**Subscription** Information about subscribing to _The Journal of Immunology_ is online at:
http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Fas/Ligand, Caspase-8 and Caspase-3 Pathways

Tyrosine Kinase Can Mediate Apoptosis of T Cells through the Fas/Fas Ligand, Caspase-8 and Caspase-3 Pathways

Lingwen Zhong, Chun-Hua Wu, Wen-Hui Lee, and Chih-Pin Liu

The TCR ζ-chain-associated protein of 70 kDa (ZAP-70), but Not Syk, Tyrosine Kinase Can Mediate Apoptosis of T Cells through the Fas/Fas Ligand, Caspase-8 and Caspase-3 Pathways

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by National Institutes of Health Grant AI44413 (to C.-P.L.) and a Cancer Center Core Grant CA33752.

2 Address correspondence and reprint requests to Dr. Chih-Pin Liu, Division of Immunology, Beckman Research Institute, City of Hope, 1450 East Duarte Road, Duarte, CA 91010-3000. E-mail address: cliu@coh.org

3 Abbreviations used in this paper: PTK, protein tyrosine kinase; ZAP-70, ζ-associated protein of 70 kDa; SH2, Src homology 2; ITAM, immunoreceptor tyrosine-based activation motif; AICD, activation-induced cell death; Fasl, Fas ligand; Erk, extra-cellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine.

C.-P.L.) and a Cancer Center Core Grant CA33752.

Received for publication May 13, 2003. Accepted for publication November 19, 2003.

The Division of Immunology, Beckman Research Institute, City of Hope, Duarte, CA 91010


Copyright © 2004 by The American Association of Immunologists, Inc.
0022-1767/04/$02.00
repertoire (28, 29). TCR engagement triggers the signals that initiate and regulate the AICD. For example, Jurkat T cells expressing a TCR-β mutant that does not bind to the TCR/CD3 ζ-chain are defective in apoptosis and Fas ligand (FasL) expression. These results suggest that TCR-mediated apoptosis requires not only signals mediated through the ζ-chain but also the association of ZAP-70 to TCR complexes (30). Furthermore, ZAP-70-deficient T cells were unable to up-regulate FasL expression and undergo AICD, indicating that ZAP-70 plays an important role in the process of AICD (31). However, the underlying mechanisms through which ZAP-70 mediates AICD remain unknown. It is also not clear whether Syk, when compared with ZAP-70, has a similar or different effect on the AICD of T cells. Studies to determine the effect of ZAP-70 and Syk on AICD of T cells have been complicated by the need of ZAP-70 or Syk to associate with receptor effect of ZAP-70 and Syk on AICD of T cells have been complicated by the need of ZAP-70 or Syk to associate with receptor.

To activate the cells, 1 × 10^5 transfectants and the BWZ cells were cultured in 96-well plates with or without plate-coated anti-TCR Ab H57 (50 μg/ml). After stimulation for various times, cells were harvested and stained with different Abs by incubating cells at room temperature or 4°C for 30 min. FITC-conjugated or biotinylated anti-hamster IgG and streptavidin-PE were used in some stainings as the secondary Ab. At least 10,000 events/sample were collected using FACSCalibur and analyzed with CellQuest (BD Biosciences, San Jose, CA).

IL-2 secretion was determined by MTT assay using the HT-2 cell line as the indicator as previously described (33).

Cell death analyses

Cells (1 × 10^5 well) were activated in 96-well plates coated with H57 or the anti-Fas Ab, Jo2, at the concentration of 50 μg/ml, or were activated with serially diluted Abs from 100 μg/ml. The percentage of apoptotic cells was measured by staining annexin V and propidium iodide (PI) (BD PharMingen). As indicated in the text, 5 μg/ml sFasFc protein were also added 30 min before activation of the cells to block the Fas/Fasl-induced cell death. Specific cell death was calculated as follows: specific cell death = (percentage of activated cell death - percentage of nonactivated cell death)/100 - percentage of nonactivated cell death) × 100.

Western blot analyses

Cytosolic extracts were prepared by lysing cells at 4°C in lysis buffer (20 mM Tris, 1 mM EDTA, 150 mM NaCl, 5 mM iodoacetamide, 1 mM Na3VO4, 1% Triton X-100 or Brij 35, 1 mM PMSF, small peptidase inhibitors). Cell lysates were then loaded onto a 10% SDS-PAGE and separated proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was blocked in PBS with 10% dry milk and 0.1% Tween 20, and incubated with primary Abs followed by an HRP-conjugated secondary Ab. The target proteins were visualized using an enhanced chemiluminescence kit (Pierce, Rockford, IL).

Measurement of caspase-3 and caspase-8 activation

The cells containing an active form of caspase-3 and caspase-8 were detected using caspTag caspase activity kit (Serologicals, Norcross, GA) according to the manufacturer’s instruction with minor changes. Briefly, cells were cultured in the presence or absence of plate-bound H57 for 4 or 24 h at 37°C, and then were stained with the fluorescence-conjugated caspase-3 inhibitor FAM-DEVd-FMK or the caspase-8 inhibitor FAM-LETD-FMK for 1 h at 37°C. After washing, cells were directly analyzed using FACS.

Results

Generation of stable transfectants expressing chimeric ZIZAP or ZSyk molecules

To study and compare the roles of ZAP-70 and Syk kinases in T cell activation and AICD, we generated DNA constructs encoding ZIZAP or Z/Syk chimeric molecules. ZIZAP or Z/Syk was generated by covalently linking a truncated TCR ζ-chain to the linker and kinase domain of ZAP-70 or Syk, respectively (Fig. 1A). Therefore, SH2-truncated ZAP-70 or Syk molecules became part of the ζ-chain in the TCR/CD3 complex and did not need to associate with other receptor ITAMs. Because the SH2 domain of ZAP-70 or Syk may block their kinase activities (7, 22–24), it was expected that the ZIZAP and Z/Syk kinase activities were not negatively regulated in the absence of the two SH2 domains. We also generated a DNA construct encoding a truncated TCR ζ-chain (Zt) that lacked all three cytoplasmic ITAMs. This allowed us to distinguish the effect of ZIZAP or Z/Syk chimeric molecules on T cell functions from tyrosine phosphorylation of GST-cdb3 was detected by immunoblotting with the anti-phosphotyrosine Ab, 4G10. The GST-cdb3 was a generous gift from Dr. R. L. Wange and L. E. Samelson (National Institutes of Health, Bethesda, MD) (36, 37).

T cell activation and flow cytometry analyses

In vitro kinase assay

In vitro kinase assays were performed by incubating whole cell lysates or 100. In some experiments, the inhibitors piceatannol or PP2 were added to reduce the reactions. Reactions were terminated by the addition of sample buffer and proteins were separated in a 10% SDS-PAGE. Tyrosine phosphorylation of GST-cdb3 was detected by immunoblotting with the anti-phosphotyrosine Ab, 4G10. The GST-cdb3 was a generous gift from Dr. R. L. Wange and L. E. Samelson (National Institutes of Health, Bethesda, MD) (36, 37).
that of endogenous ZAP-70. A cDNA encoding a full-length TCR ζ-chain consisting of an extracellular domain (EC), a transmembrane domain (TM), and a cytoplasmic domain (Cyt) with the three ITAMs. Zt was a truncated TCR ζ-chain containing EC, TM, and Cyt region without the three ITAMs. The ZZAP and Z/Syk chimeric molecules contained the same truncated ζ-chain as that of Zt, which was then covalently linked in frame to the SH2-truncated linker and kinase domain of ZAP-70 or Syk, respectively. B, TCR expression was restored on the surface of transfectants. TCR ζ-chain-deficient BW5147 cells were transfected with Zf, Zt, ZZAP, or Z/Syk DNA constructs. Surface expression of TCR on the stable transfectants was determined by staining with H57 (darker solid curve on the right). The parental BW5147 (BWZ) cells were used as the negative control (light colored curve on the left). C, Expression of transfected chimeric protein ZZAP or Z/Syk in the BWZ cells was detected by Western blot analysis of the whole cell lysates with anti-ZAP-70 or anti-Syk Abs. Loading control studies showed that the same amount of β-actin was present in each sample analyzed per lane (data not shown). D, The TCR complexes were immunoprecipitated from cell lysates of BWZ, ZZAP, Z/Syk, Zt, or Zt cells, using H57. The presence of ZZAP or Z/Syk molecules in the immunoprecipitated proteins was detected by anti-ZAP-70 or anti-Syk Abs. Additional normalization control studies were performed by stripping the membrane and immunoblotting with the anti-TCR β-chain Ab, H197. The results showed that the same amount of TCR proteins were present in each lane on the gel. The results were representative of at least five different experiments.

FIGURE 1. Generation and biochemical analyses of stable transfectants. A, Schematic representation of constructs encoding Zf, Zt, ZZAP, and Z/Syk molecules. Zf was a full-length TCR ζ-chain consisting of an extracellular domain (EC), a transmembrane domain (TM), and a cytoplasmic domain (Cyt) with the three ITAMs. Zt was a truncated TCR ζ-chain containing EC, TM, and Cyt region without the three ITAMs. ZZAP and Z/Syk chimeric molecules contained the same truncated ζ-chain as that of Zt, which was then covalently linked in frame to the SH2-truncated linker and kinase domain of ZAP-70 or Syk, respectively.

Phosphorylation and kinase activity of ZZAP and Z/Syk kinases

We next determined whether the ZZAP and Z/Syk kinases are phosphorylated in nonactivated transfectant cells and whether their phosphorylation status changes after TCR engagement. The BWZ, ZZAP, Z/Syk, Zt, and Zt cells were stimulated with an anti-CD3ε mAb (2C11) for 5 min, and the TCR complexes were immunoprecipitated with the anti-TCR β-chain Ab H57. The same amount of immunoprecipitated protein from these different cell lines was loaded on SDS-PAGE and immunoblotted with anti-phosphotyrosine Ab, 4G10. The results showed that ZZAP, rather than the endogenous ZAP-70 kinase, was immunoprecipitated with H57 (Fig. 2A). The results also showed that, after immunoprecipitation with H57, the phosphorylation of ZZAP kinase before and after TCR engagement was not detectable using 4G10 (Fig. 2A). However, when we first immunoprecipitated the tyrosine-phosphorylated proteins using 4G10 and then immunoblotted these proteins with an anti-ZAP-70 Ab, we could detect a significant amount of...
Phosphorylation of Z/ZAP and Z/Syk kinases increased after cells were activated through TCRs. A. The BWZ, Z/ZAP, Z/Syk, Zf, and Zt cells were activated by the anti-CD3ε mAb (2C11) at 37°C for 5 min, and their TCR complexes were immunoprecipitated with H57. The phosphorylation of Z/ZAP and Z/Syk kinases was detected using anti-phosphotyrosine Ab, 4G10. The amount of total Z/ZAP or Z/Syk protein was detected by anti-ZAP-70 or anti-Syk Ab. The numbers shown below the panel of 4G10 blotting results represent the relative levels of phosphorylated Z/Syk kinase. No phosphorylated Z/ZAP kinase was detected. The relative levels of phosphorylated Z/Syk kinase was calculated as the following: the levels of relative phosphorylation of Z/Syk = the density of phosphorylated Z/Syk/the density of the total amount of Z/Syk protein. B. These five cell lines were activated by 2C11 for 5 min, and the cell lysates from these cells were first immunoprecipitated by 4G10. The presence of phosphorylated Z/ZAP kinase was then detected using an anti-ZAP-70 Ab.

Because Z/ZAP and Z/Syk kinases lack the two SH2 domains of ZAP-70 or Syk, respectively, it is likely that the kinase activity of the chimeric molecules are not negatively regulated by the SH2 domains. Therefore, we studied whether Z/ZAP and Z/Syk kinases possessed kinase activities in nonactivated cells and whether their kinase activities were changed in activated cells. First, we determined the kinase activities of Z/ZAP and Z/Syk kinases in nonactivated cells. The results showed that the cytoplasmic domain of the band 3 protein (cdbc3), an exogenous substrate for ZAP-70 and Syk (36, 37), was phosphorylated only by cell lysates of nonactivated Z/ZAP or Z/Syk cells, but not by those of BWZ, Zf, or Zt cells (Fig. 3A). Furthermore, the kinase activities of Z/ZAP and Z/Syk could be inhibited by piceatannol, an inhibitor specific for Syk and ZAP-70 kinases, but not by PP2, an inhibitor for other Src family tyrosine kinases (Fig. 3B and C). As shown in Fig. 3B, the kinase activity of Z/ZAP was alleviated with the presence of 25 μg/ml piceatannol and was completely blocked at 50 μg/ml. In contrast, PP2 failed to inhibit the Z/ZAP kinase activity even at a concentration of 1 μg/ml (its IC50 for inhibiting Lck or Fyn kinase activity is 1.5 ng/ml (42)). Similarly, the kinase activity of Z/Syk cells could be inhibited only by piceatannol but not by PP2 (Fig. 3C). It was not clear whether the piceatannol concentration used to block the kinase activity correlated directly with the relative strength of ZAP-70 and Syk kinase activities. Previous studies have shown that activation of endogenous ZAP-70 was dependent on other kinases such as Lck and Fyn, thus PP2 should be able to block the kinase activity of endogenous ZAP-70 (21, 23, 25, 26). However, the failure of PP2 to block the kinase activity of Z/ZAP and Z/Syk cells suggest that Z/ZAP and Z/Syk did not require Src kinases for their kinase activities. Therefore, these results indicate that the kinase activity of Z/ZAP cells was due to the chimeric Z/ZAP kinase but not due to the endogenous ZAP-70 and Src family kinases. Similarly, the kinase activity of Z/Syk cells was due to the chimeric Z/Syk kinase rather than the other endogenous kinases. These data also show that deletion of the two N-terminal SH2 domains enable ZAP-70 and Syk molecules to display kinase activities.

Second, we determined whether the kinase activities of Z/ZAP and Z/Syk were changed after TCR activation. We performed in vitro kinase assays using proteins immunoprecipitated from cell lysates with H57. The results showed that cdbc3 was phosphorylated by proteins immunoprecipitated from Z/ZAP and Z/Syk cells, but not by those from BWZ, Zf, or Zt cells (Fig. 4). The results also confirmed the data shown in Fig. 3 that Z/ZAP and Z/Syk chimeric molecules possessed kinase activities in nonactivated cells. In addition, in nonactivated cells, the Z/Syk kinase showed higher kinase activity (>5-fold more) than that of Z/ZAP kinase, consistent with previous studies showing that the kinase activity of Syk was higher than that of ZAP-70 (20). After activation of Z/ZAP cells with 2C11, the phosphorylation of cdbc3 increased ~2.6-fold, compared with that before activation (Fig. 4). The results also showed that the Z/ZAP kinase was phosphorylated in the

**FIGURE 3.** The Z/ZAP and Z/Syk kinases possessed kinase activity in nonactivated cells. A. Kinase activity of Z/ZAP and Z/Syk was assessed by in vitro kinase assay using cell lysates of untreated transfectant cells and the BWZ cells. Phosphorylation of the cytoplasmic domain of band 3 protein (GST-cdbc3) by the various cell lysates was detected by immunoblotting with the anti-phosphotyrosine Ab, 4G10. B. Analyses of the kinase activities of Z/ZAP and Z/Syk in the presence of kinase inhibitors. The cell lysate of Z/ZAP cells was either treated with the Syk family kinase inhibitor piceatannol (IC50 for Syk family kinase was 2.44 μg/ml) at the concentrations of 10, 25, 50, and 100 μg/ml (upper panel), or with the Src family kinase inhibitor PP2 (IC50 = 1.5 ng/ml) at the concentrations of 0.09 and 1 μg/ml (lower panel). The cell lysates of BWZ, Zf, and Zt cells were used as the controls. C. The cell lysate of Z/Syk cells was treated with piceatannol at 10, 25, 50, 100 μg/ml and PP2 at 1 μg/ml. Loading control studies showed that the same amount of β-actin was present in each sample analyzed per lane (data not shown).
immunoprecipitates, perhaps due to autophosphorylation in the kinase assay. In contrast, we could not detect phosphorylation of endogenous ZAP-70 kinase in the proteins immunoprecipitated from cell lysates of all five different cells before and after they were activated. This was probably because, under the experimental conditions, immunoprecipitation of the TCR complexes using H57 Ab did not coprecipitate the endogenous ZAP-70 which could not be detected using an anti-ZAP-70 Ab (Fig. 2A). Therefore, both the kinase activity and phosphorylation of endogenous ZAP-70 were not detected in the in vitro kinase assay (Fig. 4). These results indicated that the phosphorylation of cdb3 was due to the Z/ZAP and Z/Syk kinases rather than the endogenous ZAP-70 kinase. These results also indicated that the kinase activity of Z/ZAP increased in activated cells. In comparison, the Z/Syk kinase displayed a higher basal level of kinase activity in phosphorylating cdb3 than that of Z/ZAP cells before activation (Fig. 4). Furthermore, phosphorylation of cdb3 by the Z/Syk proteins immunoprecipitated from Z/Syk cells did not change after the cells were activated. Therefore, the Z/ZAP kinase but not the Z/Syk kinase could increase its kinase activity after the cells were activated. However, the Z/Syk kinase exhibited a stronger kinase activity than that of the Z/ZAP kinase from activated Z/ZAP cells.

We next studied whether activation of these cells could induce protein phosphorylation. As shown in Fig. 5, essentially no new phosphorylated protein was detected in BWZ cells after stimulation with H57. Both Z/ZAP and Zf cells induced a similar pattern of phosphorylated proteins, but Z/ZAP cells had relatively more amounts of phosphorylated proteins at 2 min after activation and the phosphorylation of proteins sustained longer than that of Zf cells. Interestingly, Z/Syk cells not only showed different protein phosphorylation patterns as compared with that of the other cells, but they also had the most induced phosphorylated proteins than the other cells within 10 min after stimulation. These results suggest that signals mediated through Z/Syk are qualitatively different from the signals mediated through Z/ZAP or Zf and can result in more tyrosine phosphorylated proteins.

Expression of CD69 and production of IL-2 in activated cells

We then wanted to determine whether these transfected cells could respond to stimulation and up-regulate surface CD69 expression and produce IL-2. Therefore, we analyzed the expression of CD69, an early activation marker for T cells, on these transfected cells at 24 h after activation by H57 (Fig. 6A). The results showed that the surface expression of CD69 was up-regulated in all transfected cells as compared with that observed in the BWZ cells. The CD69 expression on activated Z/ZAP cells was comparable to that observed in activated Zf cells, and was higher than that observed in activated Zt cells. The results also showed that Z/Syk cells weakly up-regulated CD69 expression as compared...
with BWZ cells, and the level of increased CD69 expression was lower \( p < 0.01 \) than that observed in Zt cells.

In addition, we also determined whether these cells could produce IL-2. The results showed that Z/ZAP cells produced slightly more IL-2 than did Zt cells but the difference was not statistically significant, and that Zt cells produced significantly less IL-2 than did these two cell lines (Fig. 6B). In comparison, both Z/Syk cells and BWZ cells failed to produce a detectable amount of IL-2. Altogether, these results demonstrated that Z/ZAP cells, like Zt cells, could respond to stimulation, and up-regulate a higher level of CD69 and produce a significant amount of IL-2 after activation. Although Z/Syk cells also showed a weak CD69 up-regulation response they did not produce detectable amounts of IL-2, suggesting that the expression of the Z/Syk but not the Z/ZAP kinase may delay or even inhibit the signals that regulate the activation of T cells.

**Increased AICD of Z/ZAP but not Z/Syk cells**

Activated T cells undergo AICD which plays a key role in regulating the immune response and in shaping the peripheral T cell repertoires (28, 29, 43, 44). Signals mediated through TCR complexes play critical roles in regulating AICD of T cells (45, 46). Because the signals mediated through Z/ZAP and Z/Syk kinases are probably different from each other, we determined the effect of Z/ZAP and Z/Syk kinases on AICD of the cells expressing these molecules. The results showed that, after activation of the cells through their TCRs, the percentage of apoptotic Z/ZAP cells was markedly increased at 12 h after activation (Fig. 7, A and C). In comparison, the cell death of ZI cells, but not the other cells, slightly increased. After 24 h, as shown in Fig. 7, B and C, the death of ZI and Zt cells increased, but it was still significantly less than that of Z/ZAP cells (Fig. 7C, Z/ZAP vs ZI, \( p < 0.05 \); Z/ZAP vs Zt, \( p < 0.01 \)). Although our biochemical studies showed that the Z/Syk kinase possessed stronger kinase activity than did Z/ZAP kinase, and more phosphorylated proteins were present in Z/Syk cells after TCR activation than in Z/ZAP cells, apoptosis of Z/Syk cells did not increase relative to that of BWZ cells. The hyporeactivity of Z/Syk cells to cell death was not due to anergy because addition of IL-2 to cell culture did not increase the death of activated Z/Syk cells (data not shown). These results suggest that Z/ZAP cells can undergo apoptosis more rapidly and to a higher degree than the other cells tested. The very weak, if any, apoptotic response of activated Z/Syk cells suggests that Z/Syk kinase-mediated signaling pathways may prevent the cell death process or fail to connect the TCR-mediated pathways to the cell death pathways.

**Involvement of Fas/FasL pathway in AICD of Z/ZAP but not Z/Syk cells**

Previous studies have shown that a major apoptosis pathway of T cells is due to the interaction between Fas and Fas ligand (FasL) (28, 29, 43, 44, 47). To determine whether the Fas/FasL pathway plays a role in the apoptosis of Z/ZAP and Z/Syk cells, we first examined the cell surface expression of Fas and FasL on cells with or without the activation by H57. The results showed that Z/ZAP cells increased their FasL expression for \( \sim 12 \)-fold at 6 h after activation. The FasL expression reached its highest level (\( \sim 40 \)-fold) at 12 h and then decreased thereafter (Fig. 8A and Table I). Compared with BWZ cells, the other cells also increased their FasL expression but the fold-increase was not as fast and big as that seen in Z/ZAP cells (Table I). Although Z/Syk cells increased their FasL expression (\( \sim 6 \)-fold) at 6 h, no further increase was detected on these cells at 12 and 24 h.

Further studies on Fas expression showed that, unlike FasL, Fas was constitutively expressed on all the tested cells. However, compared with BWZ and the other cells, Z/ZAP cells expressed a higher basal level (\( \sim 1.29 \)-fold) of Fas before activation (Fig. 8B and Table I). The Fas expression on Z/ZAP cells, but not on the other cells, further increased nearly 3-fold at 12 h after activation (Fig. 8B and Table I). Interestingly, Z/Syk cells not only expressed

![Graph](http://www.jimmunol.org/)

**FIGURE 7.** Induction of activation-induced cell death. A and B, Dose response of TCR-mediated apoptosis of Z/ZAP, Z/Syk, ZI, Zt, and BWZ cells at different time points after activation. The transfectants and BWZ cells were stimulated by plate-coated H57 Ab at a 5-fold serial dilution from 200 to 0.4 \( \mu \text{g/ml} \) for (A) 12 h and (B) 24 h, respectively. The activated cells were stained with annexin V and positive cells were considered as apoptotic cells. Cells treated with isotype Ab in the absence of the H57 treatment were used as the nonactivated negative control. Specific cell death = (percentage of activated cell death − percentage of nonactivated cell death)/100 − percentage of nonactivated cell death) \( \times 100 \). C, A bar-chart analysis of TCR-mediated apoptosis of cells at different time points after activation. Cells were stimulated by plate-coated H57 at the concentration of 50 \( \mu \text{g/ml} \). After activation for 12 and 24 h, the cells were analyzed by annexin V staining. The data were the average \( \pm \) SEM of at least six independent experiments. The data represent the results that have already subtracted the results from the negative control. *, \( p < 0.05 \) when compared with BWZ cells at 12 or 24 h (Student’s \( t \) test).
showed that sFas:Fc could significantly inhibit the apoptosis of Z/ZAP cells at 24 h (Fig. 8D). In contrast, perhaps because Z/Syk cells did not show significant apoptosis following activation, sFas:Fc has no detectable effect on the apoptosis of Z/Syk cells. In addition, sFas:Fc was able to inhibit the TCR-induced apoptosis of Z and Zt cells at 24 h after activation, suggesting that the Fas/FasL-mediated cell death pathway also played a major role in AICD of these cells. Collectively, these results demonstrated that the Fas/FasL pathway could mediate the apoptosis of TCR-activated Z/ZAP cells, although other molecules or pathways might also play a role. However, these cell death pathways, including the Fas/FasL pathway, might have been blocked in Z/Syk cells.

**Rapid activation of caspase-3 and caspase-8 in Z/ZAP cells but not in Z/Syk cells after TCR stimulation**

Previous studies have clearly shown that caspases, a family of cysteine proteases, play essential roles at various stages of apoptosis (51–54). Caspases, such as caspase-3 and caspase-8, may play a role. However, these cell death pathways, including the Fas/FasL pathway, might have been blocked in Z/Syk cells.

**Table I. Fold-increase of FasL and Fas surface expression after TCR engagement for various time**

<table>
<thead>
<tr>
<th></th>
<th>FasL</th>
<th>Fas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>6 h</td>
</tr>
<tr>
<td>BWZ</td>
<td>1.00</td>
<td>0.92</td>
</tr>
<tr>
<td>Z/ZAP</td>
<td>1.13</td>
<td>1.34</td>
</tr>
<tr>
<td>Z/SYK</td>
<td>1.23</td>
<td>6.05</td>
</tr>
<tr>
<td>Z t</td>
<td>1.23</td>
<td>8.10</td>
</tr>
<tr>
<td>Z t</td>
<td>1.07</td>
<td>5.38</td>
</tr>
</tbody>
</table>

a The data in the table = staining intensity of FasL or Fas of activated cells/ staining intensity of FasL or Fas of unactivated BWZ cells. The number was the average value of at least four different experiments.

![Graph](http://www.jimmunol.org/)

**FIGURE 8.** Involvement of Fas/FasL-mediated death pathway in the activation-induced cell death of Z/ZAP, Zf, Zt cells, but not in Z/Syk and BWZ cells. A and B, Kinetic analysis of Fas and FasL surface expression on activated cells. The cells were cultured with 50 μg/ml plate-coated H57 Ab for 6, 12, and 24 h, or treated with isotype control Ab. The surface expression of (A) FasL or (B) Fas was detected by staining the cells with an anti-FasL mAb (MFL4) or anti-Fas mAb (Jo2), respectively. The staining intensity was measured by flow cytometry. The data shown represent the average ± SEM of at least three independent experiments. C, The apoptotic response of activated cells to anti-Fas Ab, Jo2, which could induce apoptosis of cells expressing Fas (20, 25, 26). The cells were first stimulated with plate-coated H57 (50 μg/ml) for 6 h, and then with different concentrations of anti-Fas Ab (Jo2) from 50 to 0.1 μg/ml. After incubation for 6 h with Jo2, cell death was analyzed by annexin V staining. D, The effect of sFas:Fc on blocking activation-induced cell death. Cells were incubated with or without the sFas:Fc protein (5 μg/ml) for 30 min, then stimulated with plate-coated H57 (50 μg/ml) for 24 h, and the cell apoptosis was measured by annexin V staining using FACS. The data represent the results that have already subtracted the results from the negative control. *, p < 0.01 compared with cells cultured in the absence of the sFas:Fc.
also play important roles in the AICD of T cells (55, 56). Caspase-8 is mainly involved in a death receptor-mediated apoptosis pathway, such as the Fas/FasL pathway and the TNFR pathway, and it can promote the cleavage and activation of a series of downstream caspases, including caspase-3 (57, 58). Caspase-3, which can also be activated by death receptor-mediated pathways or by the mitochondria/caspase-9-mediated apoptotic pathway, appears to be essential for the completion of the destructive stage of cell death (54). Therefore, to further determine the mechanisms underlying the difference of AICD between Z/ZAP and the other cells, we investigated whether caspase-3 and caspase-8 were activated and thus were involved in the AICD of Z/ZAP, Z/Syk, Zf, Zt, and BWZ cells. These cells were incubated in the presence or absence of plate-bound H57 for 4 or 24 h. The presence of the activated form of the two caspases in cells was determined by staining the cells with fluorescence-conjugated inhibitors of caspase-3 (FAM-DEVD-FMK) or caspase-8 (FAM-LETD-FMK), which specifically bind to the active form of caspase-3 or caspase-8, respectively. The fluorescence intensity of cells stained by these inhibitors was determined by FACs and represented the amount of the activated form of caspase-3 or caspase-8 present in the cells. The results showed that, after 4 h stimulation, the percentage of Z/ZAP cells containing the activated form of caspase-3 and caspase-8 increased significantly (~4.4-fold and ~3.6-fold, respectively) (Fig. 9 and Table II). In contrast, for BWZ, Z/Syk, Zt, and Zf cells, the percentage of cells containing active caspase-3 and caspase-8 remained low and was essentially unchanged at 4 h. After 24 h, the percentage of Z/ZAP cells containing active caspase-3 and caspase-8 further increased (~7.8- and ~7.9-fold, respectively) (Fig. 9 and Table II). The percentage of cells containing active caspase-3 and caspase-8 also significantly increased in Zf cells (~4.3- and ~4.6-fold, respectively) and Zt cells (~3.7- and ~3.2-fold, respectively), but the percentage did not significantly change in Z/Syk cells. These results demonstrated that both caspase-3 and caspase-8 are probably involved in the induction of rapid cell death of Z/ZAP cells, and also in the death of Zf and Zt cells. In addition, these results suggest that the failure to induce AICD of the Z/Syk cells is at least partly due to the lack of activation of caspase-3 and caspase-8 in these cells.

**Figure 9.** Rapid activation of caspase-3 and caspase-8 in Z/ZAP cells, but not in Z/Syk cells. BWZ, Z/ZAP, Z/Syk, Zf, or Zt cells were incubated without or with plate-bound H57 Ab for 4 and 24 h, or with an isotype control Ab. The percentage of cells containing an activated form of (A) caspase-3 and (B) caspase-8 were determined by fluorescence-conjugated caspase-3 or caspase-8 inhibitors. Values shown are the mean ± SEM of data obtained from at least three experiments. The data represent the results that have already subtracted the results from the negative control. *p < 0.05 compared with cells cultured in the absence of H57 (~H57). **, p < 0.05 compared with cells cultured with H57 for 4 h (+H57, 4 h).

<table>
<thead>
<tr>
<th></th>
<th>Caspase-3</th>
<th>Caspase-8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>4 h</td>
</tr>
<tr>
<td>BWZ</td>
<td>1.00</td>
<td>0.92</td>
</tr>
<tr>
<td>Z/ZAP</td>
<td>1.00</td>
<td>4.35</td>
</tr>
<tr>
<td>Z/Syk</td>
<td>1.00</td>
<td>1.07</td>
</tr>
<tr>
<td>Zf</td>
<td>1.00</td>
<td>1.02</td>
</tr>
<tr>
<td>Zt</td>
<td>1.00</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Table II. Fold-increase of the percentage of the cells with activated caspase-3 and -8 after TCR engagement for various time points

**Discussion**

The major purpose of this study is to determine the relative roles of ZAP-70 and Syk kinases in the activation and the AICD of T cells in vitro. Our results showed that Z/ZAP and Z/Syk kinases were present in the TCR complexes of the transfected cells, and the expression of Z/ZAP and Z/Syk molecules could restore the TCR expression on TCR ζ-chain-deficient cells. The results also showed that the Z/Syk kinase was phosphorylated in nonactivated cells and phosphorylation of Z/Syk increased after the cells were activated through their TCRs. In comparison, phosphorylation of the Z/ZAP kinase was much weaker in nonactivated cells but it was increased.
Our results also showed that the kinase activity of both ZAP-70 and Syk negatively regulate their kinase activity. Removal of the SH2 domains could thus enable endogenous kinases in the cells responsible for the phosphorylation of cdb3. Therefore, these results indicate that Z/ZAP and Z/Syk kinases rather than the endogenous kinases in the cells were responsible for the phosphorylation of cdb3, an exogenous substrate for ZAP-70 or Syk kinases. These results demonstrated that Z/ZAP and Z/Syk molecules possessed some kinase activity before TCR activation. Their kinase activity could be blocked by the Syk and ZAP-70 kinase inhibitor piceatannol but not by PP2, a Src kinase inhibitor. Previous studies have shown that endogenous ZAP-70 kinase activity was dependent on Src kinases such as Lck and Fyn (21, 23, 25, 26). Because PP2 did not block the kinase activity of Z/ZAP and Z/Syk cells, their kinase activity was not dependent on Src kinases. In addition, although all of five cell lines expressed the endogenous ZAP-70 kinase, only Z/ZAP and Z/Syk but not BWZ, Zf, and Zt cells showed kinase activity in phosphorylating cdb3. Therefore, these results indicate that Z/ZAP and Z/Syk kinases rather than the endogenous kinases in the cells were responsible for the phosphorylation of cdb3. Removal of the SH2 domains could thus enable ZAP-70 and Syk kinases to phosphorylate their substrates, consistent with the results of previous studies that the two SH2 domains of both ZAP-70 and Syk negatively regulate their kinase activity (7, 22–24). Our results also showed that the kinase activity of Z/ZAP from activated Z/ZAP cells increased ~2.6-fold relative to that of nonactivated cells, consistent with its significantly increased phosphorylation level. In contrast, the Z/Syk kinase showed a higher basal level of phosphorylation and its phosphorylation increased ~2.8-fold in activated cells compared with that of nonactivated cells. However, the kinase activity of Z/Syk did not change in activated cells, although the Z/Syk kinase activity was higher than that of Z/ZAP kinase and more proteins were phosphorylated in Z/Syk cells than in Z/ZAP and other cells within 10 min after activation. Therefore, the results demonstrated that Z/ZAP and Z/Syk kinases might behave differently from each other in T cells.

An interesting finding in this report is that Z/ZAP and Z/Syk kinases play different roles in regulating AICD of T cells. Although both Z/ZAP and Z/Syk kinases possessed kinase activity in nonactivated cells, they did not cause detectable cell death in these cells. This may be because their kinase activity was below the threshold required to induce cell death. However, our results also demonstrated that stimulation of the cells through TCRs led to more rapid cell death response and a higher percentage of apoptotic Z/ZAP cells than even the positive control Zf cells. It has been shown previously that defective recruitment of ZAP-70 to the TCR complex inhibited the induction of FasL expression and apoptosis of the cells (30, 59). Our data were consistent with these findings and further demonstrated that the expression of Z/ZAP kinase in the TCR complex resulted in a rapid increase of the Fas and FasL expression and the AICD of Z/ZAP cells compared with the other cells. The results that sFas:Fc could block the death of Z/ZAP cells demonstrate that the Fas/FasL-mediated cell death pathway is involved in the apoptosis of the cells. In addition to Fas/FasL, it has been shown that TNFR1 (p55) and TNFR2 (p75) can regulate the apoptosis of T cells (28, 29, 50, 60, 61). Therefore, we performed studies to determine whether the TNF-α receptor (TNFR)-mediated pathway played a role in causing AICD of Z/ZAP cells. Our results showed that, although expression of the two TNFRs was increased on activated Z/ZAP cells, neither the neutralizing Abs against these two receptors nor the soluble TNFR:Fc protein was able to rescue activated Z/ZAP cells from apoptosis (data not shown). This demonstrated that the TNFR-mediated pathway did not play a role in the apoptosis of the Z/ZAP cells.

It has been shown that both caspase-3 and caspase-8 play critical roles in mediating apoptosis of cells (55, 56). In our studies to further determine the mechanisms leading to the rapid death of activated Z/ZAP cells, we investigated the roles of caspase-3 and caspase-8.
caspase-8. We found that the activation of Z/ZAP cells, but not other cells, quickly induced the activation of both of these caspases. Therefore, it is likely that the activation of caspase-3 and caspase-8 was involved in the apoptosis of activated Z/ZAP cells. Treatment of Z/ZAP cells with sFas:Fc could reduce the activation of both caspase-3 and caspase-8, and it has a stronger blocking effect on the activation of caspase-8 than caspase-3. These results suggest that the Z/ZAP kinase can stimulate multiple pathways, including the Fas/FasL pathway, that result in the activation of caspase-3 and caspase-8 and the induction of rapid cell death following TCR activation. In addition, we also studied whether p38, extracellular signal-regulated kinase (Erk), and c-Jun N-terminal kinase (JNK) kinases were involved in the cell death process in Z/ZAP cells (data not shown). We found that the phosphorylation status of p38 and Erk did not change significantly after the cells were activated, suggesting that both of these two kinases were not involved in the apoptosis of Z/ZAP cells. In contrast, JNK was phosphorylated in activated Z/ZAP cells more quickly than in other cells. Previous studies have shown that JNK might be involved in apoptosis of cells. For example, JNK-deficient T cells exhibited reduced AICD (62), and the defective apoptosis of JNK-deficient murine embryonic fibroblasts was caused by the failure of mitochondria cytochrome c release and caspase-3 activation (63). Therefore, it is likely that phosphorylation of JNK in Z/ZAP cells may facilitate activation of caspase-3, and eventually lead to cell death.

In contrast to that of Z/ZAP cells, activated Z/Syk cells did not increase Fas expression and did not contain the activated form of caspase-3 and caspase-8. They also showed little, if any, detectable levels of AICD. In contrast, Z/Syk cells responded equally well to a cell death-inducing anti-Fas Ab, Jo2, and underwent apoptotic death. These results suggest that the difference in AICD between Z/Syk cells and Z/ZAP cells, as well as other cells, was not due to a defective or altered Fas/FasL pathway in Z/Syk cells. It was more likely to be due to the difference of signals mediated through TCRs containing either the Z/ZAP or Z/Syk kinases. The observation that Z/Syk cells failed to demonstrate an increased activation and AICD was unexpected. One would expect to see a stronger effect of Z/Syk kinase on AICD than did Z/ZAP kinase because it has been shown previously that the Syk kinase had stronger kinase activity than ZAP-70 kinase does (20, 25, 26). However, the apoptotic response of Z/Syk cells to TCR activation was weaker than that of not only Z/ZAP cells but also Zt cells. Although Z/Syk cells up-regulated a lower level of CD69, produced little IL-2, and showed a weaker apoptotic response to activation, more and probably different tyrosine phosphorylated proteins were induced in Z/Syk cells than in Z/ZAP and other cells. Altogether, these results suggest that the signals mediated through Z/Syk kinase could inhibit the AICD of T cells rather than that the cells were simply unresponsive to activation through their TCRs.

The current studies provide an interesting view of the potential roles of ZAP-70 and Syk in T cell development and function. Previous studies have shown that, while both Syk and ZAP-70 played critical roles in early thymocyte development, ZAP-70 played a more important role in regulating the development and function of matured thymocytes and T cells (3, 5, 64, 65). This difference may be partly due to the lower expression level of Syk in these cells. However, although Syk is not expressed at high levels in many peripheral T cells, it is expressed at high levels in thymocytes and in peripheral effector T cells. For example, it has been shown that peripheral T cells bearing high levels of Syk are present in ZAP-70-deficient humans (19). In addition, a recent report has demonstrated that the ζ-chain in human effector cells can be replaced by the FcRγ chain, which then recruits Syk rather than ZAP-70 in activated cells (66). Therefore, Syk may play a critical role in the generation of immune responses mediated by these effector cells. Although Syk−/− mice contain normal T cells, Syk is critical to the normal development of T cells as demonstrated in studies of Syk/ZAP-70 double-deficient mice. The thymocyte development in these Syk/ZAP-70 double-deficient mice is blocked at the CD4+ CD8+ thymocyte stage, whereas the thymocyte development in ZAP-70 single-deficient mice is blocked at the more mature CD4+ CD8+ thymocyte stage. Therefore, while most T cells express lower levels of Syk, some naive T cells and effector T cells do express Syk at higher levels. These results suggest that Syk and ZAP-70 can play some nonredundant role in T cell development.

It remains unknown why Syk and ZAP-70 are expressed at different levels in thymocytes and in different populations of T cells (14, 34). Our results that ZAP-70 and Syk play different roles in activation and AICD should be helpful to elucidate some important characteristics regarding the functional differences of these cells. Based on our results, it is possible that expression of Syk at a lowered level in matured T cells is partly due to the fact that the Syk kinase has a negative effect on regulating T cell activation and AICD. Therefore, thymocytes using Syk as the major signaling molecules are likely to be developmentally disadvantaged in comparison with the cells using ZAP-70. Thus, it is likely that ZAP-70 and Syk play different roles in shaping the selection of a normal T cell repertoire. They may also contribute differently to the generation of normal T cell immunity, e.g., against tumor cells. It has recently been shown that Syk regulates the growth of tumor cells, such as breast cancer cells (67, 68). Expression of Syk in a breast cancer cell line can suppress malignant growth of the cells rather than cause apoptosis of the tumor cells in athymic mice. Our results are consistent with the results from these cancer cell studies and further suggest that Syk may also play a more regulatory role in the function and growth of T cells. Therefore, Syk and ZAP-70 are expected to play different roles in regulating various T cell behaviors. We are in the process of generating and characterizing transgenic mice expressing Z/ZAP or Z/Syk kinases. Future studies on these animals should provide us with further information of the in vivo roles of these kinases on the function and development of T cells.

Acknowledgments
We thank Drs. A. Chan, C.-L. Law, and B. Malissen for generously providing us with DNA and cell reagents.

References


43. M-B cell receptor complex.


