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Requirement for Shc in TCR-Mediated Activation of a T Cell Hybridoma

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Engagement of the TCR determines the fate of T cells to activate their functional programs, proliferate, or undergo apoptosis. The intracellular signal transduction pathways that dictate the specific outcome of receptor engagement have only been partially elucidated. The adapter protein, Shc, is involved in cytokine production, mitogenesis, transformation, and apoptosis in different cell systems. We found that Shc becomes phosphorylated on tyrosine residues upon stimulation of the TCR in DO11.10 hybridoma T cells; therefore, we investigated the role of Shc in activation-induced cell death in these cells by creating a series of stably transfected cell lines. Expression of Shc-SH2 (the SH2 domain of Shc) or Shc-Y239/240F (full-length Shc in which tyrosines 239 and 240 have been mutated to phenylalanine) resulted in the inhibition of activation-induced cell death and Fas ligand up-regulation after TCR cross-linking. Expression of wild-type Shc or Shc-Y317F had no significant effect. In addition, we found that Shc-SH2 and Shc-Y239/240F, but not Shc-Y317F, inhibited phosphorylation of extracellular signal-regulated protein kinase and production of IL-2 after TCR cross-linking. These results indicate an important role for Shc in the early signaling events that lead to activation-induced cell death and IL-2 production after TCR activation. The Journal of Immunology, 1999, 163: 2586–2591.

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Materials and Methods

Abs and reagents

The Abs used in this study include anti-mouse IL-2, mouse anti-hamster cross-linking Ab, and PE-conjugated anti-CD95 (clone MFL3; PharMin- gen, San Diego, CA); anti-LAT and anti-PLCγ1 (6E-152; Upstate Biotechnol- ogy, Lake Placid, NY); anti-mouse CD3ε (145-2C11) and anti- phospho- MAPK (New England Biolabs, Cambridge, MA); anti-Grb2 (Santa Cruz Biotechnology, Santa Cruz, CA); and anti-Shc (Transduction Laboratories, Lexington, KY). Anti-phosphotyrosine Ab (4G10) was a gift from Dr. Tom Roberts (Dana-Farber Cancer Institute, Boston, MA). 51 Cr was purchased from New England Nuclear (Boston, MA). Propidium io- dide (PI) and PMA were obtained from Sigma (St. Louis, MO). Ionomycin was purchased from Calbiochem (San Diego, CA). Murine Fasl-Fc fusion protein was provided by Dr. Shyr-Te Ju (Boston University Hospital, Boston, MA).

Plasmids

The GST-Shc constructs used in these studies were generated by PCR and subcloned into the pEBG vector, as described previously (8, 9).

Cells and transfections

The murine T cell hybridoma, DO11.10 (10), was provided by Dr. Barbara Osborne (University of Massachusetts, Amherst, MA). The B cell hybridi- oline, LK 35.2 (11), was obtained from Dr. Christoph Klein (Children’s Hospital, Boston, MA). Cells were grown in RPMI 1640 containing 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. Cells (106) were transfected at 800 μf/250 V using a BRL (Gaithersburg, MD) electroporator. Twenty micrograms of each of the GST-Shc constructs were transfected. Geneticin-resistant transfectants were tested for expression of the Shc construct by Western blot analysis.

T cell activation

For phosphorylation studies, cells were incubated with anti-CD3 Ab (1 μg/ml) for 10 min on ice followed by a 10-min incubation on ice with anti-hamster cross-linking Ab. Samples were subsequently incubated for 2 min at 37°C. For IL-2 production and apoptosis studies, cells were stim- ulated by plate-bound anti-CD3 Ab for the indicated times.

Immunoprecipitations and immunoblotting

Unstimulated or stimulated cells were lysed in buffer containing 0.5% Triton X-100, 50 mM Tris (pH 7.6), 150 mM NaCl, 1 mM Na3V4O7, 10 mM NaF, 10 mM sodium pyrophosphate, and protease inhibitors. Lysates were subjected to immunoprecipitation with anti-Shc Abs and protein A-Sepha- rose or glutathione-Sepharose beads. For phospho-MAPK studies, total cell lysates from 1 × 106 cells were analyzed. Proteins were subjected to SDS-PAGE separation, transferred to a polyvinylidene difluoride membrane, and immunoblotted with the appropriate Abs.

IL-2 production assays

IL-2 production was measured by a standard ELISA according to the manu- facturer’s protocol (Genzyme, Cambridge, MA). Cells were stimulated for 12 h, as indicated in the figure legends, and plates were read at 450 nm on a Bio-Rad (Richmond, CA) plate reader.

Apoptosis assays

Cells were left unstimulated or were stimulated with plate-bound anti-CD3 for the indicated periods of time. Apoptotic cells were analyzed by flow cytometry after staining with hypotonic PI solution as described previously (12).

FasL-mediated lysis assays

The functional activity of FasL was determined by the ability of FasL-expressing cells to induce apoptosis in Fas+ LK35.2 target cells as de- scribed previously (13). Briefly, 5 × 106 LK 35.2 cells were labeled for 1 h at 37°C with 20 μCi 145 Cr. Stably transfected DO11.10 cells were incubated (104 cells/well) for 3 h at 37°C in anti-CD3-coated 96-well plates before 104 145 Cr-labeled LK 35.2 cells were added. In FasL blocking studies, acti- vated DO11.10 cells were incubated with 10 μg/ml murine FasL-Fc fusion protein before incubation with the LK35.2 target cells. After an additional 6-h incubation, 100 μl of supernatant was removed from each well and counted in a gamma counter to determine experimental release. The percent specific lysis was calculated with the following formula: % lysis = 100 × [(experimental release − spontaneous release)/(total release − spontaneous release)].
The TCR (data not shown) and mutant Shc proteins (Fig. 1) were generated and analyzed. Expression levels of both wild-type and mutant Shc tyrosines are responsible for Shc function during T cell activation. Several clones that stably express the wild-type and mutant Shc proteins were chosen for specific reasons. In epidermal growth factor receptor signal transduction studies, the SH2 domain of Shc (Shc-SH2) has been shown to function as a dominant interfering protein that blocks endogenous Shc function (15, 17, 18). The tyrosine mutants were designed because Y239, Y240, and Y317 constitute the three major phosphorylation sites on Shc following TCR stimulation (19). Since tyrosine phosphorylation of Shc is important for its interaction with Grb2 and for mediating its downstream signaling effects (18, 20), the Shc-Y317F and Shc-Y239/240F mutants were expected to elucidate which, if any, of these interactions mediated by Y239/240 of Shc.

Expression of dominant interfering Shc mutants inhibits apoptosis in DO11.10 hybridoma cells

DO11.10 cells undergo apoptosis in response to TCR-mediated stimulation. If Shc is essential for mediating early TCR-initiated signaling events, the effect of the dominant interfering mutants could be manifested by their influence on AICD. To test this possibility, the stable cell lines expressing mutant Shc proteins were examined for their ability to undergo AICD upon TCR cross-linking. Representative clones with high stable expression of each of the transfected genes were analyzed by PI staining after 8 or 12 h of stimulation with anti-CD3 Ab (Fig. 2A). Flow cytometric analysis of the subdiploid peak, which represents cells undergoing apoptosis, revealed that cells that expressed Shc-Y317F underwent apoptosis to an extent comparable to cells that express wt-Shc in response to anti-CD3 cross-linking. In contrast, expression of dominant-interfering Shc-SH2 or Shc-Y239/240F markedly inhibited apoptosis. In all experiments, <5% of the unstimulated cells underwent apoptosis. To establish that these differences weren’t due to clonal variation, at least five clonal cell lines expressing each Shc construct were tested for their ability to undergo AICD after 12 h of anti-CD3 cross-linking. The pooled results are shown in Fig. 2B. These data strongly suggest that Shc plays a critical role in early signaling events that involve the Shc-SH2 domain and interactions mediated by Y239/240 of Shc.

Expression of Shc-SH2 and Shc-Y239/240F inhibits expression of FasL

AICD in DO11.10 cells (and many other T cells) occurs through the up-regulation of FasL, on the activated cells and the subsequent interaction of FasL with Fas expressed on the same or neighboring cells (reviewed in Ref. 21). The Fas:FasL interaction leads to suicide or fratricide through the ensuing Fas-mediated death pathway. To determine whether the inhibition of apoptosis mediated by mutant Shc proteins is due to the inhibition of FasL up-regulation, we used a sensitive bioassay to determine the effect of Shc on FasL that relies upon the ability of FasL+ cells to lyse 51Cr-labeled Fas-sensitive LK35.2 cells (13). Fig. 3 shows that Fas-mediated cytolyis of LK35.2 cells was induced during AICD of DO11.10 cells, and this cytolyis was not affected by the expression of wt-Shc. This result indicates that DO11.10 cells up-regulate their FasL expression during AICD. The cytolytic effect of DO11.10 cells on LK35.2 was completely inhibited by preincubation of the DO11.10 cells with murine Fasl-Fc fusion protein, indicating that the killing was mediated by the Fas-Fasl interaction (data not shown). FasL expression was inhibited by >50% with expression

![Figure 2](image-url)

**FIGURE 2.** Dominant interfering forms of Shc have an anti-apoptotic effect. A, The effect of expression of Shc mutants on apoptosis was determined by flow cytometric analysis of cells treated with hypotonic PI 8 or 12 h after anti-CD3 cross-linking as described in Materials and Methods. B, Data from several experiments, using at least five individual clonal cell lines for each Shc mutant after stimulation for 12 h, are expressed as the percent apoptosis. Error bars indicate SEs. Statistical analyses were performed using the nonparametric Mann-Whitney U test. Differences between DO11.10 and Shc-SH2 (p < 0.0015) and DO11.10 v. Shc-Y239/240F (p < 0.0001) are statistically significant.

![Figure 3](image-url)

**FIGURE 3.** Shc-SH2 and Shc-Y239/240F inhibit expression of FasL. Cells were stimulated for 3 h by anti-CD3 cross-linking and were subsequently incubated for 6 h with Fas+ 51Cr-labeled LK35.2 target cells. Assays were performed in triplicate. 51Cr release, which indicates Fas-mediated lysis of target cells, was measured in a gamma counter. One representative experiment of three performed is shown.
of Shc-SH2 or Shc-Y239/240F, but not Shc-Y317F. Stimulation of the cells with PMA plus ionomycin, which bypasses TCR stimulation and Shc activation, resulted in comparable induction of FasL cell surface expression (42–54%) in all stable cell lines analyzed (data not shown). The inhibition of FasL expression by mutant Shc proteins correlates with the effects of these proteins on apoptosis of DO11.10 cells. These results suggest that Shc plays an important role in TCR-induced up-regulation of FasL and the subsequent induction of apoptosis.

TCR-dependent IL-2 production is regulated by Shc

Concomitant with the induction of apoptosis, TCR engagement of DO11.10 cells leads to the secretion of IL-2. To determine whether Shc plays a role in the events that lead to IL-2 production, cells were stimulated for 12 h by anti-CD3 cross-linking. A standard ELISA was performed, and plates were read at 450 nm to detect IL-2 production. Pooled results from four separate experiments are shown. B, IL-2 production of cells stimulated with PMA plus ionomycin. Error bars indicate SEs. Differences between DO11.10 and Shc-SH2 (p < 0.05) and between DO11.10 and Shc-Y239/240F (p < 0.0047) are statistically significant.

Mutation of Y239/240F of Shc inhibits interaction with Grb2

Many of the functional effects of Shc are mediated by interaction of Shc with Grb2 and activation of the Ras signaling pathway. Shc contains two potential binding sites for Grb2 within its CH domain: Y239/240 and Y317. Stable Shc-transfected DO11.10 cell lines were tested for the association of endogenous Grb2 with GST-Shc by anti-Grb2 immunoblotting of proteins that precipitated with glutathione-Sepharose beads. As shown in Fig. 5A, anti-TCR cross-linking led to the association of Grb2 with wt-Shc. A comparable amount of Grb2 was associated with Shc-Y317F. In contrast, very little Grb2 associated with Shc-Y239/240F. This finding suggests that Y239/240 of Shc is the major Grb2 binding site in TCR-stimulated DO11.10 cells. The inability of Grb2 to bind to Shc-Y239/240F may play a role in the inhibitory effects observed in cells expressing this protein.

Cross-linking the TCR on DO11.10 cells also induces phosphorylation of tyrosine residues of several other signaling proteins, including PLCγ1 and LAT. To demonstrate the specificity of the effect of Shc mutants on signaling pathways, we analyzed the phosphorylation status of these two proteins in the different stable cell lines in response to TCR cross-linking. Expression of dominant-interfering Shc proteins had no effect on the phosphorylation state of PLCγ1 (Fig. 5B) and LAT (Fig. 5C).

Mutation of Y239/240F of Shc inhibits phosphorylation of ERK

Many of the functional effects of Shc observed in cell systems are mediated by activation of the Ras-Raf-MEK1/2-ERK1/2 signaling pathway is bypassed (Fig. 4B). These data demonstrate that Shc plays a role in TCR-mediated synthesis of IL-2, and this is dependent upon Shc-SH2 and the phosphorylation of Shc on Y239/240 residues.
pathway. ERK activation is correlated with phosphorylation on tyrosine residues, which can be detected by an Ab that specifically recognizes tyrosine-phosphorylated ERK1 and ERK2 (tyrosine 204). To determine whether activation of ERK correlated with the functional effects observed by expression of mutant Shc proteins in T cells, phosphorylation of ERK in the cell lines that express Shc proteins was assessed. As shown in Fig. 6, anti-TCR cross-linking led to robust phosphorylation of ERK in DO11.10 cells and cell lines with stable expression of Shc-wt and Shc-Y317F. In contrast, expression of Shc-SH2 and Shc-Y239/240F inhibited phosphorylation of ERK in response to anti-CD3 stimulation. This finding suggests that the inhibitory effect of Shc-SH2 and Shc-Y239/240F on IL-2 production and AICD in TCR-stimulated DO11.10 cells is mediated through inhibition of the ERK pathway. Confirmation of this finding was evidenced by analysis of the effect of Shc mutants on activation of MEK, an upstream activator of ERK. Expression of Shc-SH2 and Shc-Y239/240F inhibited phosphorylation of MEK in response to anti-CD3 cross-linking. In contrast, expression of Shc-wt and Shc-Y317F had no effect (data not shown).

Discussion

Shc was initially identified as an oncogene that induced a transformed phenotype in cultured fibroblasts and tumor formation in nude mice (1). Since then, the role of Shc in epidermal growth factor signaling and mitogenesis as well as its role in the regulation of cell cycle progression and integrin signaling have been well characterized (22, 23). An anti-apoptotic role for Shc in IL-3 withdrawal-induced apoptosis of the murine pro B cell line BaF3 has been described (3). In contrast, our studies demonstrate a positive role for Shc in the early signaling events that lead to AICD. Although the pathways involved in apoptosis in these two systems have not been completely elucidated, it has become increasingly clear that distinct mechanisms regulate apoptosis in different cell types. Cytokine withdrawal-mediated apoptosis does not activate the Fas pathway, whereas AICD involves Fas-mediated apoptosis. Several studies have shown that the up-regulation of FasL is pivotal for AICD and is dependent on Lck, ZAP-70, CD45, calcineurin, and Ras activities and an intact CD3-ζ (24–28). Subsequent to its up-regulation, FasL interacts with Fas and activates the Fas death pathway.

Our data suggest that Shc is required for the up-regulation of FasL during AICD. The contradictory roles of Shc in cytokine withdrawal-mediated apoptosis and AICD further emphasize the differences in these two mechanisms of apoptosis. A similar contradictory role for c-Myc in apoptosis has been described. c-Myc serves as a survival factor in cytokine withdrawal-induced apoptosis, whereas FasL expression during AICD requires c-Myc expression (29). We found that expression of dominant interfering Shc-SH2 or Shc-Y239/240F diminished FasL up-regulation as determined by a Fas-mediated cytolysis assay, indicating that Shc affects signaling events upstream of FasL expression. We did not find an inhibitory effect by mutation of Y317 of Shc, which suggests that the signaling pathway elicited through this tyrosine is not involved in these functional events. This was surprising, as many of the previous studies on the function of Shc had attributed its effects to its interaction with Grb2 through Y317. However, a role for Grb2 in the Shc functions described here cannot be ruled out, as it has also recently been shown that Grb2 can bind to Shc through tyrosine 239 and lead to the formation of a Shc:Grb2: mSos complex (19, 30). In our studies we have found that mutation of Y317 did not diminish the amount of Grb2 that associated with Shc, while mutation of Y239/240 severely blocked this association. This finding correlates with the inhibition of apoptosis, IL-2 production, and up-regulation of FasL activity observed in cells that express Shc-Y239/240F, but not Shc-Y317F. The Shc-SH2 construct is also unable to bind to Grb2, but can still potentially interact with other critical signaling molecules. Therefore, the inability to form a productive signaling complex that involves an Shc-Grb2 association results in the dominant negative effects observed by expression of the Shc mutants. This Shc-Grb2 complex is necessary for phosphorylation of MEK and its substrate, Erk, which, in turn, are required for downstream signaling events. As expected, we did not detect an effect of mutant forms of Shc on phosphorylation of PLCγ (Fig. 5B) or LAT (Fig. 5C), indicating that the effect of the Shc mutants on T cell signaling is specific for the Grb2-Mek-MAPK pathway.

There has been much controversy regarding the functional role of Shc in T cells. Although reports have demonstrated phosphorylation of Shc in response to TCR activation and the association of Shc with TCR-ζ (4–6), studies in Jurkat T cells have failed to confirm these results. Our data in DO11.10 cells clearly indicate that Shc plays a role in early TCR-mediated signaling events that ultimately result in AICD and IL-2 production. This is in apparent contrast to reports that Shc is not involved in TCR-stimulated NF-AT activation in Jurkat T cells (15, 31). We have also been unable to detect involvement of Shc in IL-2 production in two distinct Jurkat cell lines (data not shown). We believe that this may be due to poor tyrosine phosphorylation of Shc in Jurkat cells following TCR/CD3 stimulation, in contrast to the robust phosphorylation of Shc that can be detected in normal peripheral blood T cells and several other murine and human T cell lines. In addition to the detection of tyrosine-phosphorylated Shc in anti-TCR stimulated DO11.10 cells (Fig. 1A), we have seen robust phosphorylation of Shc in CD4+ splenocytes under conditions that induce AICD (16) (data not shown).

Expression of the dominant interfering Shc constructs, Shc-SH2 and Shc-Y239/240F, inhibited FasL expression, apoptosis, and IL-2 production by approximately 50% compared with that in parental DO11.10 cells or cells expressing Shc-wt or Shc-Y317F constructs. One possible explanation why a higher degree of inhibition was not observed in these assays is that expression of the dominant interfering constructs did not completely block the ability of endogenous Shc to form productive signaling complexes. Alternatively, it is possible that an Shc-independent signaling pathway contributes to the regulation of Ras activation and subsequent IL-2 production and AICD. LAT is one candidate molecule that has also been shown to play a role in the recruitment of a Grb2: mSOS complex and activation of NF-AT in T cells (32, 33). We did not find an effect of expression of Shc mutants on anti-CD3-stimulated tyrosine phosphorylation of PLCγ (Fig. 5B) or LAT (Fig. 5C); therefore this signaling pathway remains intact in these cells and may contribute to the IL-2 production, apoptosis, and FasL expression that are not inhibited by the dominant interfering...
Promoter elements identified to date that regulate the FasL promoter


