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Signal-Transducing Adaptor Protein-2 Modulates Fas-Mediated T Cell Apoptosis by Interacting with Caspase-8

Yuichi Sekine,* Chikako Yamamoto,* Michinori Kakisaka,* Ryuta Muromoto,* Shigeyuki Kon,* Dai Ashitomi,* Natsuko Fujita,† Akihiko Yoshimura,† Kenji Oritani,† and Tadashi Matsuda*

We found that an adaptor protein, signal-transducing adaptor protein (STAP)-2, is a new member of the Fas–death-inducing signaling complex and participates in activation-induced cell death in T cells. STAP-2 enhanced Fas-mediated apoptosis and caspase-8 aggregation and activation in Jurkat T cells. Importantly, STAP-2 directly interacted with caspase-8 and Fas, resulting in enhanced interactions between caspase-8 and FADD in the Fas–death-inducing signaling complex. Moreover, STAP-2 protein has a consensus caspase-8 cleavage sequence, VEAD, in its C-terminal domain, and processing of STAP-2 by caspase-8 was crucial for Fas-induced apoptosis. Physiologic roles of STAP-2 were confirmed by observations that STAP-2–deficient mice displayed impaired activation-induced cell death and superantigen-induced T cell depletion. Therefore, STAP-2 is a novel participant in the regulation of T cell apoptosis after stimulation. The Journal of Immunology, 2012, 188: 6194–6204.

The online version of this article contains supplemental material.

Materials and Methods
Reagents, Abs, and mice

Recombinant human TRAIL, PHA, and dexamethasone (DEX) were obtained from Wako (Osaka, Japan). Recombinant human TNF-α was a gift from Dainippon Sumitomo Pharma (Osaka, Japan). Recombinant murine His-tagged FasL was purchased from R&D Systems (Minneapolis, MN). Propidium iodide, staurosporine, and etoposide were purchased from Sigma-Aldrich (St. Louis, MO). The pan-caspase inhibitor z-VAD-fmk and the caspase-8-specific inhibitor z-IETD-fmk were purchased from Calbiochem (Darmstadt, Germany). Expression vector for Halo-tagged human Fas and HaloLink resin were purchased from Promega (Madison, WI). Expression vectors for HA-tagged caspase-8/Mch5 (17) and FLAG-FADD

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The number of viable cell was analyzed using CellTiter-Glo transfectants were also cultured without or with the indicated amount of iodide and Annexin-V-Fluos (Roche, Penzberg, Germany) or TUNEL Mixture [Wako]). Extracts from control groups (anti–Fas-untreated cell). In vitro caspase-8 digestion assay, 293T cells were transfected with Myc-tagged STAP-2. At 36 h after transfection, the cells were lysed and immunoprecipitated with anti-Myc Ab. The immunoprecipitates were soaked with caspase-8 assay buffer (20 mM PIPES, pH 7.2, 0.1 M NaCl, containing 0.1% CHAPS and 10% sucrose, 10 mM DTT, 1 mM EDTA, 1 mM PMSF) and incubated with recombinant active caspase-8 (5 U; MLB, Tokyo, Japan) at 37 C for 4 h. After boiling the SDS sample buffer, samples were subjected to SDS-PAGE, followed by Western blot analysis.

FACS analysis

The following mAbs were used: PE-conjugated anti-B220, FITC–anti-CD4, PE–anti-CD4, PE–anti-CD95, FITC–anti-CD178, FITC–anti-TCR-β, FITC–anti-V β6 and FITC–anti-V β8 Abs (BD Pharmingen). Cell suspensions were incubated for 30 min on ice with an appropriate Ab in 50 μl PBS. The two-color cytofluorometric analysis was performed as described above.

Transfection of small interfering RNA

Silencing of caspase-8 or caspase-3 expression in Jurkat/STAP-2 cell was achieved by their specific small interfering RNAs (siRNAs). Jurkat/STAP-2 cells (5 x 10⁶) were nucleofected with control (Qiagen) or caspase-8 (no. 1: 5'-GAGUGUGAGGCUUAAUCAATTT-3', no. 2: 5'-UGUAGUUGCUUGCCUACUAUAAAT-3', no. 3: 5'-UAUGUGUGAUUAGUUAGCUAGGAGTT-3') or caspase-3 (no. 1: 5'-GGCUUGAUGUUGCUUGAGCUAAAC-3', no. 2: 5'-UAUGUGUCUUGCUUAGCUAAUAAAT-3', no. 3: 5'-UGUAGUUGCUUGCCUACUAUAAAT-3') siRNA using the Cell Line Nucleofector Kit V (Ammax Biosystems, Gaithersburg, MD).

In vivo mouse model for AIID

The mouse model of in vivo AIID was performed as described previously (23). In brief, anti-CD3 Ab (145-2C11) (5 μg) or isotype control Ab (5 μg) was injected i.v. into the tail veins of 9–11-wk-old WT or STAP-2 knockout (KO) mice. Then, 20 h later the splenocytes were isolated from the mice and stained with FITC–anti-TCR-β (BD Pharmingen) and PE–anti-B220 (BD Pharmingen) Abs. The ratio of T cells (TCR-β) to B cells (B220) was determined by flow cytometry. The number of apoptotic cells was determined by TUNEL assay using In Situ Cell Death Detection Kit, TMR Red (Roche Diagnostics) by FACS.

Staphylococcal enterotoxin B-induced T cell deletion in vivo

WT or STAP-2 KO mice (10 wk old) were treated i.p. with 2 μg staphylococcal enterotoxin B (SEB) (Toxin Technology) in 200 μl PBS on days 1, 3, and 5. On days 0 and 7, peripheral blood taken from the tail vein was stained with PE–anti-CD4 and FITC–anti-V β6 (clone RR4-7; BD Pharmingen). The percentage of SEB-responsive (V β6+) CD4+ T cells were analyzed by flow cytometry.

Apoptosis of OVA-reactive CD4+ T cells

Detection of apoptotic OVA-reactive CD4+ T cells was described previously (22). Mice were immunized s.c. in the hind footpads with 100 mg chicken OVA (Sigma-Aldrich) emulsified in CFA (Wako; OVA/CFA). For the indicated periods after OVA/CFA immunization, popliteal lymph node cells were stained with FITC–anti-CD4 and PE–anti-CD11c Abs and analyzed by FACS.
We observed significantly enhanced cell death in Jurkat/STAP-2 cells after PHA treatment compared with empty vector-expressing Jurkat T cells (Jurkat/pcDNA3 cells; Fig. 1A). Next, we examined the involvement of STAP-2 in DR-mediated cell death. Fas-mediated cell death was induced in Jurkat T cells by exposure to a Fas agonist mAb. Jurkat/STAP-2 cells displayed higher susceptibility to cell death than Jurkat/pcDNA3 cells. Similarly, Jurkat/STAP-2 cells showed enhanced cell death in response to TNF-α and TRAIL, although their susceptibilities to cell death were lower than that for Fas stimulation. We then examined the involvement of STAP-2 in the Bim-dependent mitochondrial apoptotic pathway in T cells. We used etoposide and staurosporine as Bim-dependent apoptotic stimuli. No significant differences in cell death in response to these drugs were observed between STAP-2-overexpressing and control Jurkat T cells (Supplemental Fig. 1A). Thus, the enhanced cell death displayed in the STAP-2 transfectants is selective for the DR-mediated apoptotic pathway.

**FIGURE 1.** STAP-2 enhances Fas-mediated apoptosis and caspase activation in Jurkat T cells. (A) Jurkat/pcDNA3 (filled circle), Jurkat/STAP-2#1 (filled triangle) or Jurkat/STAP-2#2 (filled square) cells (2×10^4/well) were cultured in 96-well plates in the absence or presence of the increasing amount of PHA (24 h), anti-Fas Ab (24 h), TNF-α (48 h) or TRAIL (48 h). The cell viability was measured using a Cell Counting Kit-8. The data are the means of triplicate experiments, which generally varied by ±10%. Similar results were obtained in three independent experiments. (B) Jurkat/pcDNA3 or Jurkat/STAP-2 cells (1×10^6) were stimulated without (+) or with (+) anti-Fas Ab (10 ng/ml). At 12 h after stimulation, apoptotic cells were determined by flow cytometry using TUNEL-staining. Similar results were obtained in three independent experiments. (C) Jurkat/pcDNA3 or Jurkat/STAP-2 cells (1×10^6) were stimulated with anti-Fas Ab (10 ng/ml) for the indicated periods. The cells were then lysed, and total cell lysate (TCL) was immunoblotted (IB) with anti-caspase-8, anti-cleaved caspase-8, anti-caspase-3, anti-cleaved caspase-3, anti–caspase-7, anti-cleaved caspase-7, anti-PARP, anti-cleaved PARP, anti-Myc, or anti-actin Ab. (D) Jurkat/pcDNA3 or Jurkat/STAP-2 cells (1×10^6) were stimulated with increased amounts of anti-Fas Ab (0, 1, 5, 10 ng/ml). The cells were then assessed for caspase-8 (3 h after stimulation) or caspase-3/7 (5 h after stimulation), and activity was analyzed as described in Materials and Methods. Data are expressed relative to the value of control samples without Fas stimulation. Results are representative of three independent experiments, and the error bars represent the SD of means. (E) Jurkat/STAP-2 cells (2×10^5/well) were cultured in 96-well plates without or with anti-Fas Ab (0.5 ng/ml) and increased concentrations of z-VAD-fmk (0.5, 1 μM) or z-IETD-fmk (20, 40 μM) for 24 h. The cell viability was measured using a Cell Counting Kit-8. Similar results were obtained in three independent experiments. Data are the means of triplicate experiments, and the error bars represent the SD of means. *p < 0.05 (Student t test).
Increased caspase activation through Fas in STAP-2-expressing Jurkat T cells

We focused on effects of STAP-2 on Fas-mediated apoptosis, because Fas and FasL have a major role in AICD of T cells. To determine whether STAP-2 affected the susceptibility of Jurkat cells to Fas-mediated cell death, apoptosis of the stable transfectants was evaluated with TUNEL staining (Fig. 1B) as well as annexin V staining (Supplemental Fig. 1B) after the treatment with an anti-Fas mAb. Enhanced induction of apoptosis upon Fas stimulation was detected in Jurkat/STAP-2 cells. We then analyzed the activation of caspases after Fas stimulation. As shown in Fig. 1C, the cleavage of caspase-3, -7 and -8 as well as that of PARP was significantly increased in Jurkat/STAP-2 cells compared with control cells. These findings were confirmed by observations that Fas stimulation markedly increased the caspase enzymatic activities in Jurkat/STAP-2 cells (Fig. 1D), although slightly enhanced basal caspase activation was observed. To confirm the requirement for caspase-8 in the STAP-2–mediated apoptotic process, cells were cultured in the presence of a general caspase inhibitor, Z-VAD, or a caspase-8–specific inhibitor, IETD. Pre-treatment with these compounds completely blocked Fas-mediated cell death in Jurkat/STAP-2 cells (Fig. 1E). Therefore, enhanced activation of caspase-8 is a critical step in the increased susceptibility to Fas-mediated cell death exhibited in STAP-2 transfectants.

Caspase-8 is recruited to the multiprotein Fas-DISC that is rapidly formed on the cytoplasmic portion of the Fas receptor after ligand engagement by FADD. Therefore, we examined the effect
of STAP-2 on the Fas-DISC formation. Anti-Fas mAb treatment led to caspase-8 association with Fas in the Fas-DISC, and cleaved caspase-8 was produced (Fig. 2A). Importantly, the amount of caspase-8 in the Fas-DISC was remarkably increased in Jurkat/STAP-2 cells compared with control cells. The amount of FADD in the Fas-DISC was also increased in Jurkat/STAP-2 cells (Fig. 2B). Notably, STAP-2 itself existed in the Fas-DISC in Jurkat/STAP-2 cells (Fig. 2C). Moreover, the association of caspase-8 with FADD was markedly enhanced by overexpression of STAP-2 (Fig. 2D). Thus, STAP-2 is likely to enhance the Fas-DISC formation after Fas stimulation. We then examined effects of STAP-2 on caspase-8 aggregation. Aggregation of caspase-8 in the absence of 2-ME was markedly increased in 293T and Jurkat cells overexpressing STAP-2 (Supplemental Fig. 2A, 2B). These findings indicate that STAP-2 enhances caspase-8 aggregation in the Fas-DISC.

Association of STAP-2 with caspase-8

As shown in Fig. 2E, the immunoprecipitates for STAP-2 contained caspase-8 protein in samples from 293T cells expressing HA-caspase-8 and Myc-STAP2. A similar interaction between STAP-2 and caspase-8 was also observed in Jurkat/STAP-2 cells expressing Myc-tagged STAP-2 (Supplemental Fig. 2C). The immunoprecipitates for STAP-2 contained caspase-8 protein even in the absence of Fas stimulation, suggesting that the interaction between STAP-2 and caspase-8 occurred spontaneously. To confirm the endogenous association of the two proteins, we used a T cell lymphoma cell line, HUT78, which abundantly expressed both endogenous STAP-2 and caspase-8. The immunoprecipitates with the anti–caspase-8 Ab contained endogenous STAP-2 protein, whereas those with the control Ab did not (Fig. 2F). In addition, both endogenous STAP-2 and caspase-8 existed in the Fas-DISC (Supplemental Fig. 2D). Thus, STAP-2 constitutively associates with caspase-8 in cells. Next, we examined whether STAP-2 interacts with FADD, another component of the Fas-DISC. STAP-2 failed to interact with FADD in samples of 293T cells expressing FLAG-FADD and Myc-STAP2 (Supplemental Fig. 2E). Similarly, an association between STAP-2 and FADD was not observed in Jurkat/STAP-2 cells (Supplemental Fig. 2F). However, coexpression of STAP-2 with caspase-8 and FADD caused an enhanced interaction between caspase-8 and FADD, which is consistent with the data shown in Fig. 2G. These findings strongly suggest that STAP-2 has an important role in the complex formation between caspase-8 and FADD.

Next, we determined the caspase-8–interacting domain in STAP-2 using a series of Myc-tagged STAP-2 deletion mutants (Fig. 3A). Deletion mutants of the C-terminal domain or the PH domain of STAP-2 as well as full length of STAP-2 showed interactions with caspase-8, but a deletion mutant lacking the SH2 domain failed to bind to caspase-8 (Fig. 3B). To further confirm these findings, a series of deletion mutants of STAP-2 fused with GST (GST-STAP-2

**FIGURE 3.** Interacting domains of STAP-2 with caspase-8. (A) Schematic diagrams of the domain structures of the STAP-2 deletion mutant fragments, the GST-fused STAP-2 mutant fragments, and the domain structure of the caspase-8 deletion mutant fragments. (B) 293T cells (1 × 10⁶) were transfected with HA-tagged caspase-8 (8 μg) or a series of Myc-tagged STAP-2 WT or deletion mutants (10 μg). At 36 h after transfection, the cells were lysed, immunoprecipitated with anti-Myc Ab, and immunoblotted with the indicated Abs (upper panels). TCL was blotted with indicated Abs (lower panels). (C) 293T cells (1 × 10⁶) were transfected with HA-tagged caspase-8 (15 μg) and/or a series of GST-fused STAP-2 WT or mutants (10 μg). At 36 h after transfection, the cells were lysed, pulled down with glutathione-Sepharose, and immunoblotted with anti-HA or anti-GST Ab (upper panels). TCL was blotted with anti-HA or anti-GST Ab (lower panels). (D) 293T cells (1 × 10⁶) were transfected with Myc-tagged STAP-2 (8 μg) and/or a series of FLAG-tagged caspase-8 deletion mutants (15 μg). At 36 h after transfection, the cells were lysed, immunoprecipitated with anti-FLAG Ab, and immunoblotted with the indicated Abs (upper panels). TCL was blotted with indicated Abs (lower panels). (E) Jurkat transfectants (2 × 10⁶/well) were cultured in 96-well plates with the indicated amounts of anti-Fas Ab for 24 h. The cell viability was measured using a Cell Counting Kit-8. The data are the means of triplicate samples, which generally varied by <10%. Similar results were obtained in three independent experiments. (F) Jurkat transfectants (1 × 10⁶) were stimulated without or with anti-Fas Ab (10 ng/ml) for 3 h. The cells were then assessed for caspase-8 activities. Data are expressed relative to the value of control samples without Fas stimulation. Results are representative of three independent experiments, and the error bars represent the SD of means. *p < 0.05 (Student t test).
PH, GST-STAP-2 SH2, and GST-STAP-2 C) were used (Fig. 3A). The precipitates for the GST–STAP-2 SH2 protein clearly contained caspase-8 protein (Fig. 3C). However, no caspase-8 protein was detected in the precipitates for GST–STAP-2 PH protein or GST–STAP-2 C protein. These findings further indicate that the SH2-like domain of STAP-2 interacts with caspase-8. We also determined the STAP-2–interacting domain on caspase-8 using a series of caspase-8 deletion mutants (Fig. 3A). The two N-terminal death effector domain (DED) domains of caspase-8 showed obvious interactions with STAP-2 as strong as full-length caspase-8 did, whereas the catalytic domain failed to bind to STAP-2 (Fig. 3D). Therefore, caspase-8 interacts with STAP-2 through its N-terminal DED domains.

To assess the physiologic roles of the association between STAP-2 and caspase-8, we used stable Jurkat cell transfectants expressing an empty vector (pcDNA3), WT STAP-2 or STAP-2 ΔSH2, which lacks the SH2-like domain. Significant cell death in response to Fas stimulation was observed in Jurkat/STAP-2 WT cells compared with Jurkat/pcDNA3 cells (Fig. 3E). However, the Fas-induced cell death in Jurkat/STAP-2 ΔSH2 cells was moderate. We also found that the enhanced Fas-induced caspase-8 enzymatic activity was significantly reduced in Jurkat/STAP-2 ΔSH2 cells compared with Jurkat/STAP-2 WT cells (Fig. 3F). Therefore, the association of STAP-2 with caspase-8 through its SH2-like domain enhances Fas-induced cell death and caspase-8 enzymatic activation in Jurkat T cells.

Proteolytic cleavage of STAP-2 by caspase-8 is required for Fas-mediated signaling

As seen in Fig. 2A, Fas-mediated proteolytic cleavage of STAP-2 was observed in Jurkat/STAP-2 WT cells after Fas stimulation. Therefore, we focused on the potential role of STAP-2 proteolysis during Fas-mediated signaling. First, we examined the proteolytic cleavage of endogenous STAP-2 after Fas stimulation using HUT78 cells. Cleaved STAP-2 as well as cleaved caspase-8 was observed in HUT78 cells treated with the anti-Fas Ab (Fig. 4A). We then investigated which domains of STAP-2 were cleaved in response to Fas stimulation. Jurkat cells expressing a series of Myc-tagged STAP-2 deletion mutants were treated with an anti-Fas Ab. Mutants lacking the PH or SH2 domain, but not a deletion mutant for the C-terminal domain of STAP-2, successfully generated cleaved STAP-2 within 1 h after Fas stimulation (Fig. 4B), indicating that the proteolytic cleavage site of STAP-2 may exist in the C-terminal domain. To examine the involvement of caspases in the Fas-mediated processing of STAP-2, we transfected specific siRNAs for caspase-3 (nos. 1 and 2) or caspase-8 (nos. 1 and 2) into Jurkat/STAP-2 cells. Reduction in caspase-8 expression resulted in a marked decrease in STAP-2 cleavage after Fas stimulation, whereas the reduction in caspase-3 expression did not (Fig. 4C). We next tested whether recombinant active caspase-8 can cleave the STAP-2 protein in vitro. As shown in Fig. 4D, recombinant active caspase-8 cleaved the partially purified STAP-2 protein from the Myc-STAP-2–transfected 293T cells. Furthermore, Z-VAD treatment inhibited generation of the cleaved STAP-2.

FIGURE 4. Proteolytic cleavage of STAP-2 by caspase-8. (A) HUT78 cells (1 × 10⁷) were stimulated with anti-Fas Ab (100 ng/ml) for the indicated periods. The cells were then lysed, and an aliquot of total cell lysate (TCL) was immunoblotted (IB) with anti–STAP-2, anti–caspase-8 or anti-actin Ab. (B) Jurkat/STAP-2 WT, Jurkat/STAP-2 ΔPH, Jurkat/STAP-2 ΔSH2, and Jurkat/STAP-2 ΔC cells (1 × 10⁶) were stimulated with anti-Fas Ab (10 ng/ml) for the indicated periods. The cells were then lysed, and an aliquot of TCL was immunoblotted with anti-Myc or anti-actin Ab. (C) Jurkat/STAP-2 cells (5 × 10⁶) were nuclease treated with control, caspase-8#1, caspase-8#2, caspase-3#1 or caspase-3#2 siRNA (200 pmol) by nucleofection with cell line Nucleofector Kit V as described in Materials and Methods. At 36 h after nucleofection, the cells were stimulated without (−) or with (+) anti-Fas Ab (20 ng/ml) for 3 h. The cells were then lysed, and an aliquot of TCL was immunoblotted with anti-Myc, anti-caspase-8, anti-caspase-3, or anti-actin Ab. (D) 293T cells (1 × 10⁶) were transfected with Myc-tagged STAP-2 (10 μg). At 36 h after transfection, the cells were lysed and immunoprecipitated with anti-Myc Ab. The immunoprecipitates were soaked with caspase-8 assay buffer and incubated with recombinant active caspase-8 (5 U) at 37°C for 4 h in the absence or presence of Z-VAD. After boiling in the SDS sample buffer, samples were subjected to SDS-PAGE, followed by immunoblotting with anti-Myc or anti-caspase-8 Ab.
Thus, caspase-8 is required for the STAP-2 processing during Fas-mediated signaling. In general, caspases cleave their substrates specifically at the C-terminal side of aspartic acid (the P1 site), which is dictated by arginine and glutamine residues (24). Indeed, the consensus caspase-8 cleavage site was reported to be (I/L/V)EXD (25). Thus, we searched for this consensus sequence in the C-terminal domain of STAP-2, because a deletion mutant of the C-terminal domain failed to produce the cleaved fragment after Fas stimulation. We found a VEAD sequence (residues 257–260) as a potential caspase-8 cleavage site (Fig. 5A). To further evaluate the potential cleavage of STAP-2 at this site, we generated an STAP-2 mutant with substitution of alanine for aspartic acid at

**FIGURE 5.** Caspase-8–dependent STAP-2 cleavage occurs at the position of Asp260. (A) The consensus caspase-8 cleavage site in STAP-2 and the substitution of Asp (D)-260 to Ala (A) or Glu (E) in STAP-2 are schematically shown. (B) Jurkat cells (1 × 10^7) were transiently transfected with Myc-tagged STAP-2 WT, DA, or DE (10 μg), stimulated with anti-Fas Ab (20 ng/ml) for the indicated periods. The cells were lysed, and an aliquot of TCL was immunoblotted (IB) with anti-Myc or anti-actin Ab. (C) Jurkat/pcDNA3, Jurkat/STAP-2 WT, and Jurkat/STAP-2 DA cells (1 × 10^7) were stimulated with anti-Fas Ab (20 ng/ml) for the indicated periods. The cells were lysed, and an aliquot of total cell lysate (TCL) was immunoblotted (IB) with anti-caspase-8, anti-caspase-3, anti-Myc, or anti-actin Ab. (D and E) Time course analysis of Fas-DISC formation was performed as described in Materials and Methods. Jurkat/pcDNA3, Jurkat/STAP-2 WT, or Jurkat/STAP-2 DA cells (2 × 10^6) were stimulated with anti-Fas Ab (20 ng/ml) at the indicated periods. The immunoprecipitate with anti-IgM Ab was separated on SDS-PAGE and immunoblotted with anti–caspase-8 (D) or anti-FADD Ab (E, upper panel). An aliquot of TCL was blotted with anti-caspase-8 (D), anti-FADD (E), anti-Myc Ab (D, E, lower panels). The data are representative of three independent experiments. (F) Jurkat/pcDNA3, Jurkat/STAP-2 WT, or Jurkat/STAP-2 DA cells (1 × 10^7) were stimulated without or with anti-Fas Ab (10 ng/ml). The cells were then assessed for caspase-8 (3 h after stimulation) or caspase-3/7 (5 h after stimulation) activities as described in Materials and Methods. The data are indicated as a relative caspase activity from triplicate experiments, and the error bars represent the SD. *p < 0.05 (Student t test). (G) Jurkat/pcDNA3, Jurkat/STAP-2 WT, or Jurkat/STAP-2 DA cells (1 × 10^6) were stimulated without or with TNF-α (100, 500 ng/ml). The cells were then assessed for caspase-8 (3 h after stimulation) activity as described in Materials and Methods. The data are indicated as a relative caspase activity from triplicate experiments, and the error bars represent the SD. *p < 0.05 (Student t test). (H) Jurkat/pcDNA3, Jurkat/STAP-2 WT, or Jurkat/STAP-2 DA cells (1 × 10^6) were stimulated without or with TNF-α (100, 500 ng/ml). The cells were then assessed for caspase-8 (3 h after stimulation) activity as described in Materials and Methods. The data are indicated as a relative caspase activity from triplicate experiments, and the error bars represent the SD. *p < 0.05 (Student t test). (I) Jurkat/pcDNA3 (filled circle), Jurkat/STAP-2 WT#1 (filled square), Jurkat/STAP-2 DA#1 (filled triangle), or Jurkat/STAP-2 DA#2 (open triangle) cells (2 × 10^6) were cultured in 96-well plates with the indicated amounts of anti-Fas Ab (24 h). The cell viability was measured using a Cell Counting Kit-8. The data are the means of triplicate experiments, which generally varied by <10%. Similar results were obtained in three independent experiments.
Myc Ab (Fas or anti-Myc Ab) and immunoprecipitated with anti-Myc Ab and immunoblotted with anti-Halo-tagged Fas (10 kDa). DISC formation was analyzed by immunoblotting with anti-caspase-8.

The cells were lysed and immunoprecipitated with anti-Fas Ab, and the Fas-Halo-tagged proteins were pulled down with HaloLink resin and immunoblotted with anti-Myc or anti-Fas Ab (upper panels). TCL was blotted with anti-Myc or anti-HA Ab (lower panels).

residue 260 (STAP-2 DA). We also constructed another STAP-2 mutant by substitution of glutamic acid for aspartic acid at residue 260 (STAP-2 DE) to avoid the effect of alteration in local charge at residue 260. Expression vectors of STAP-2 WT, STAP-2 DA, or STAP-2 DE were transiently expressed into Jurkat cells and cells were stimulated anti-Fas mAb for the indicated periods. Although substitution of Asp260 with a neutral amino acid, Ala, differing in size and polarity, resulted in a slightly faster mobility in the SDS-PAGE gels, Fas-mediated cleavage of STAP-2 was not observed in Jurkat cells expressing either STAP-2 DE or STAP-2 DA (Fig. 5B). Therefore, Fas/caspase-8-mediated processing of STAP-2 occurs at Asp260 in its C-terminal domain.

Activation of caspase-3 and -8 was greatly increased in Jurkat/STAP-2 DA cells compared with Jurkat/STAP-2 WT cells (Fig. 5C). Furthermore, the amount of caspase-8 or FADD, or both, in the Fas-DISC was also reduced in Jurkat/STAP-2 DA cells compared with Jurkat/STAP-2 WT cells (Fig. 5D, 5E). We further examined whether enhanced enzymatic activation of caspases by STAP-2 was affected by this mutation. As shown in Fig. 5F, Fas-induced caspase-3 and -8 enzymatic activities in Jurkat/STAP-2 DA cells were markedly reduced compared with Jurkat/STAP-2 WT cells. Moreover, TNF-α-induced caspase-8 enzymatic activity in Jurkat/STAP-2 DA cells was also significantly reduced compared with Jurkat/STAP-2 WT cells (Fig. 5G), suggesting that the cleavage of STAP-2 at Asp260 by caspase-8 is required for other DR-mediated apoptotic pathways. Fas-induced apoptosis in Jurkat/STAP-2 DA cells was weaker than that in Jurkat/STAP-2 WT cells, and at a level similar to that in Jurkat/pcDNA cells (Fig. 5H, 5I). Therefore, proteolytic cleavage of STAP-2 at Asp260 by caspase-8 is required for the enhancement of Fas-induced apoptosis and caspase-8 enzymatic activation by STAP-2 in Jurkat T cells.

We examined the molecular mechanisms related to how the STAP-2 DA mutant affects the Fas-DISC formation. We tested the association of caspase-8 with STAP-2 WT and DA in 293T cells. As shown in Fig. 6A, both STAP-2 WT and DA interacted with caspase-8. Because STAP-2 interacted with Fas (Fig. 6B), we tested the association of Fas with STAP-2 WT and DA in 293T cells.

FIGURE 6. STAP-2 associates with Fas. (A) 293T cells (1 × 10⁷) were transfected with Myc-tagged STAP-2 WT or DA (6 μg) and with or without HA-tagged caspase-8 (10 μg). At 36 h after transfection, the cells were lysed and immunoprecipitated with anti-HA Ab and immunoblotted with anti-Myc or anti-HA Ab (upper panels). TCL was blotted with anti-Myc or anti-HA Ab (lower panels). (B) 293T cells (1 × 10⁷) were transfected with Myc-tagged STAP-2 WT (6 μg) and with or without Halo-tagged Fas (10 μg). At 36 h after transfection, the cells were lysed and immunoprecipitated with anti-Myc Ab and immunoblotted with anti-Fas or anti-Myc Ab (upper panels). TCL was blotted with anti-Fas or anti-Myc Ab (lower panels). (C) 293T cells (1 × 10⁷) were transfected with Myc-tagged STAP-2 WT or DA (6 μg) and with or without Halo-tagged Fas (10 μg). At 36 h after transfection, the cells were lysed and pulled down with HaloLink resin and immunoblotted with anti-Myc or anti-Fas Ab (upper panels). TCL was blotted with anti-Myc or anti-Fas Ab (lower panels).
STAP-2 KO (filled circle) mice (6–8 wk old) were immunized s.c. in the hind footpads with OVA/CFA (100 μg in CD4+ T cells) calculated according to the formula: % apoptosis = (% annexin V+ apoptotic cells at indicated day in CD4+ T cells/% annexin V+ apoptotic cells at day positive and FITC-CD4–positive cells were detected by FACS. (20 h after injection, isolated splenocytes were stained with anti–TCR-β+CD4+ Abs and analyzed by FACS analysis (Supplemental Fig. 3A, 3B). Splenic CD4+ T cells from STAP-2–deficient mice exhibited a markedly diminished ability to undergo apoptosis in response to FasL stimulation (Fig. 7A, 7B). Furthermore, the FasL-induced activation of caspase-3 and -8 was decreased in STAP-2–deficient splenic CD4+ T cells (Fig. 7C). More importantly, the FasL–induced AICD formation was also decreased in STAP-2–deficient splenic CD4+ T cells (Fig. 7D).

To examine whether STAP-2 also influences Fas-mediated T cell apoptosis in vivo, we examined AICD induced by i.v. injection of an anti-CD3 Ab. Anti-CD3 Ab injection caused T cells to undergo AICD, resulting in a reduced T cell-to-B cell ratio in spleen cells of WT mice (Fig. 8A). However, no significant reduction was observed in those of STAP-2–deficient mice. Similar results were obtained when apoptotic cells were evaluated by TUNEL staining (Fig. 8B). Finally, we treated mice with a superantigen, SEB, that leads to the elimination of Vβ8+ (superantigen responsive) T cells in a Fas-dependent manner (26). SEB injection caused STAP-2–deficient mice to display a decreased ability to eliminate Vβ8+ CD4+–responsive cells compared with WT mice (Fig. 8C), but did not lead to elimination of the Vβ6+CD4+ T cell population (unresponsive to SEB) in either WT or STAP-2–deficient mice (Fig. 8D). We further investigated the more physiologic role of STAP-2 in AICD by studying apoptosis in Ag-specific CD4+ T cells in STAP-2–deficient mice induced by immunization with OVA/CFA.

The above findings suggest that absence of STAP-2 results in longer life span of Ag-stimulated CD4+ T cells because of lowered sensitivity to AICD. Notably, the frequencies of apoptotic CD4+ T cells in the draining lymph nodes decreased after immunization in STAP-2–deficient mice (Fig. 8E). However, DEX-induced cell death in splenic T cells was not altered by STAP-2 deficiency (Supplemental Fig. 3C). In addition, DO11.10 T cells overexpressing STAP-2 responded to DEX as strong as control cells (Supplemental Fig. 3D). Therefore, these data support the notion that STAP-2 has a physiologic role in the ability of the immune system to eliminate activated T cells.

**Discussion**

The domain structure of STAP-2 contains several potential tyrosine phosphorylation sites as well as PH- and SH2-like domains, and STAP-2 shows the characteristics of an adaptor protein that can interact with several other signaling molecules. In this study, we identified new functions of STAP-2, specifically that it is a member of the Fas-DISC components and enhances Fas-mediated apoptosis in T cells. The STAP-2–mediated effect is selective for the DR-
mediated pathway because STAP-2 expression does not alter the sensitivity of Jurkat cells to Bim-dependent apoptotic stimuli. The critical target of the STAP-2–mediated effects is the activation of caspase-8, a key initiator caspase in the Fas signaling pathway. Indeed, stable expression of STAP-2 leads to a marked increase in caspase-8 activation upon Fas engagement, and the addition of a caspase-8 inhibitor blocks Fas-mediated cell death in STAP-2–transfected cells. Splenic T cells from STAP-2–deficient mice exhibit a reduced ability to undergo AICD, as well as defects in the SEB-mediated elimination of Vβ8^CD4^ T cells. These findings indicate that STAP-2 can control T cell apoptosis by modulating one of the critical initiating events in the Fas signaling cascade.

In general, caspase-8 activation within the Fas-DISC is believed to occur in two steps. For the first step, recruitment of FADD to Fas promotes dimerization and conformational changes of caspase-8, and allows caspase-8 to gain full enzymatic activity. Next, active caspase-8 undergoes autoproteolytic processing and leaves the Fas-DISC to access its substrates. Among the Fas-DISC components, FLASH associates with procaspase-8 in nonapoptotic cells (27). After Fas signaling is stimulated, FADD and then a FLASH–procaspase-8 complex are recruited into the intracellular domain of the aggregated Fas, resulting in the promotion of caspase-8 activation. Similar to FLASH, STAP-2 recognized the DED domain of caspase-8, even under steady-state conditions, and enhanced the interaction between FADD and caspase-8 as well as the aggregation and activation of caspase-8 during Fas signaling. Although STAP-2 did not bind to FADD, the molecular mechanisms for how FLASH and STAP-2 enhance Fas-induced apoptosis may be similar. For example, STAP-2 may control the local concentration and intracellular distribution of procaspase-8 before Fas stimulation. Alternatively, STAP-2 may promote a conformational change of procaspase-8 to allow dimerization or rapidly expose its autoproteolytic active sites in the Fas-DISC.

STAP-2 has a consensus caspase-8 cleavage sequence, Vead, in its C-terminal domain, and was clearly cleaved by caspase-8 after Fas stimulation. It is noteworthy that processing of STAP-2 was crucial for Fas-induced apoptosis. Consequently, STAP-2 may participate in the Fas-DISC as a suicide substrate to enhance Fas-induced apoptosis. For example, activated caspase-8 may easily leave the Fas-DISC after processing of STAP-2. Although FLASH is also proteolytically cleaved during Fas signaling, the requirement for the cleavage is likely to differ between STAP-2 and FLASH. In the case of FLASH, its C-terminal region contains a potential cleavage site for caspase-8 and a nuclear localization signal (27). Thus, the cleaved FLASH C-terminal is thought to translocate into the nucleus. Although not a Fas-DISC component, hematopoietic progenitor kinase 1 (HPK1), a mammalian Ste20-related protein kinase, is a substrate of caspase-8, a key initiator caspase in the Fas signaling pathway. Indeed, stable expression of STAP-2 leads to a marked increase in caspase-8 activation upon Fas engagement, and the addition of a caspase-8 inhibitor blocks Fas-mediated cell death in STAP-2–transfected cells. Splenic T cells from STAP-2–deficient mice exhibit a reduced ability to undergo AICD, as well as defects in the SEB-mediated elimination of Vβ8^CD4^ T cells. These findings indicate that STAP-2 can control T cell apoptosis by modulating one of the critical initiating events in the Fas signaling cascade.

Although our data are reproducible, there are some questions against these molecular events in the Fas-DISC. One question is how STAP-2 ΔSH2, lacking the binding capacity to caspase-8, was cleaved by caspase-8 (Fig. 4B). This might suggest that STAP-2 cleavage is independent on tight binding between STAP-2 and caspase-8, because both proteins may exist closely enough in the Fas-DISC. The other question is why STAP-2 DA inhibited recruitment of caspase-8 into the Fas-DISC (Fig. 5D). For the possible mechanism, the caspase-8–cleaved site might bind to another Fas-DISC component. Indeed, we showed that STAP-2 DA could recognize caspase-8, but not Fas (Fig. 6A, 6C). Because molecular events in the DISC during Fas-stimulation are complicated, further analysis will clarify these strange phenomena regarding to STAP-2.

Notably, expression of FLICE-like inhibitory protein (FLIP), an inhibitory protein of Fas-mediated apoptosis (30), was slightly decreased in Jurkat/STAP-2 cells (Supplemental Fig. 4A). Furthermore, STAP-2–deficient T cells also showed a slightly increased FLIP expression (Supplemental Fig. 4B). Thus, the involvement of STAP-2 in FLIP expression is likely to be another possible mechanism for the DISC formation, although we need more careful examinations in this regard.

Given that STAP-2 interacts with a variety of signaling molecules, an attractive hypothesis is that the presence or absence of specific partners may modulate the ability of STAP-2 to function in several signaling pathways. In this study, we identified caspase-8 as a novel partner of STAP-2 in T cells. Future investigations will clarify when and how STAP-2 acts as a regulator of caspase-8 activation in vivo.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. STAP-2 enhances Fas-mediated apoptosis in Jurkat T-cells. (A) Jurkat transfectants (2x10^5/well) were cultured in 96-well plates in the absence or presence of the increasing amount of etoposide (24 h) or staurosporine (24 h). The cell viability was measured using a Cell Counting Kit-8. The data are the means of triplicate experiments, which generally varied by <10%. Similar results were obtained in three independent experiments. (B) Jurkat/pcDNA3 or Jurkat/STAP-2 cells (1x10^6) were stimulated without or with anti-Fas antibody (10 ng/ml). At 12 h after stimulation, cells were stained with Annexin V and propidium iodide (PI) and analyzed by FACS. Numbers refer to the percentage of cells encountered in each quadrant. Similar results were obtained in three independent experiments.
Supplemental Figure 2. Dispensable binding capacity of STAP-2 to caspase-8 and FADD. (A, B) Aggregation of caspase-8 by STAP-2. (A) 293T cells (1x10^6) were transfected with HA-tagged caspase-8 (10 μg) without or with Myc-tagged STAP-2 (10 μg). At 36 h after transfection, the cells were lysed, and boiled in SDS sample buffer with (+) or without (-) 2-ME. TCL was blotted with anti-HA and anti-Myc antibodies. (B) Jurkat/pDNA3 or Jurkat/STAP-2 cells (3x10^6) were stimulated with anti-Fas antibody (20 ng/ml) for the indicated periods. The cells were lysed and boiled in SDS sample buffer without 2-mercaptoethanol. TCL was blotted with anti-caspase-8, anti-Myc or anti-actin antibody. Association of STAP-2 with caspase-8. (C) Jurkat/pDNA3 or STAP-2 cells (3x10^6) were stimulated without (-) or with (+) anti-Fas antibody (20 ng/ml) for 3 h. The cells were lysed, immunoprecipitated with anti-Myc antibody, and immunoblotted with the indicated antibodies (upper panels). TCL was also blotted with indicated antibodies (bottom panels). (D) HUT78 cells (2x10^6) were stimulated with anti-Fas antibody (100 ng/ml) at the indicated periods. The immunoprecipitate with anti-Fas antibody was separated on SDS-PAGE and immunoblotted with the indicated antibodies (upper panels). TCL was blotted with the indicated antibodies (lower panels). (E, F) STAP-2 does not associate with FADD. (E) 293T cells (1x10^6) were transfected with FLAG-tagged FADD (20 μg) without or with Myc-tagged STAP-2 (10 μg). At 36 h after transfection, the cells were lysed, and immunoprecipitated with anti-Myc antibody and immunoblotted with anti-FLAG or anti-Myc antibody (upper panels). TCL was blotted with anti-FLAG or anti-Myc antibody (lower panels). (F) Jurkat/pcDNA3 or Jurkat/STAP-2 cells (1x10^6) were lysed after stimulation at the indicated times with anti-Fas antibody (20 ng/ml). The cells were lysed, and immunoprecipitated with anti-Myc antibody and immunoblotted with anti-FADD or anti-Myc antibody (upper panels). TCL was also blotted with anti-FADD, anti-caspase-8 or anti-Myc antibody (lower panels).
Supplemental Figure 3. STAP-2 influences neither Fas/FasL expression nor Dex-induced cell death.  (A, B) Comparison of Fas and FasL expression between WT and STAP-2 KO T-cells. WT or STAP-2 KO mice were intravenously injected with anti-CD3 antibody (5 μg) or isotype control antibody (5 μg). After 20 hours, splenocytes were double-stained with anti-CD4 and anti-Fas (A) or anti-FasL (B) antibodies and analyzed by FACS. The data represents the Fas and FasL expressions in CD4 positive splenocytes. Data are from one representative experiment. Similar results were obtained in three independent experiments. (C) Dexamethasone (Dex)-induced cell death in STAP-2 KO T-cells. Splenic T-cells (1x10⁶) from WT or STAP-2 KO mice, or DO11.10 transfectants (1x10⁶) were stimulated with the increasing amounts of Dex for 48 h. The data are the means of triplicate experiments, which generally varied by <10%. Similar results were obtained in three independent experiments.
Supplemental Figure 4, STAP-2 influences FLIP expression in T-cells. (A) Jurkat transfectants (1x10⁶) were lysed and TCL was analyzed by immunoblotting (IB) with anti-FLIP (Alexis), anti-Myc or anti-actin antibody. (B) Splenic T cells (1x10⁶) from WT or STAP-2 KO mice were stimulated with sFasL for the indicated periods. The cells were then lysed and TCL was analyzed by IB with anti-FLIP (Alexis) or anti-actin antibody.