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A Fas-Associated Death Domain Protein/Caspase-8-Signaling Axis Promotes S-Phase Entry and Maintains S6 Kinase Activity in T Cells Responding to IL-2

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Fas-associated death domain protein (FADD) constitutes an essential component of TNFR-induced apoptotic signaling. Paradoxically, FADD has also been shown to be crucial for lymphocyte development and activation. In this study, we report that FADD is necessary for long-term maintenance of S6 kinase (S6K) activity. S6 phosphorylation at serines 240 and 244 was only observed after long-term stimulation of wild-type cells, roughly corresponding to the time before S-phase entry, and was poorly induced in T cells expressing a dominantly interfering form of FADD (FADDdd), viral FLIP, or possessing a deficiency in caspase-8. Defects in S6K1 phosphorylation were also observed. However, defective S6K1 phosphorylation was not a consequence of a wholesale defect in mammalian target of rapamycin function, because 4E-BP1 phosphorylation following T cell activation was unaffected by FADDdd expression. Although cyclin D3 up-regulation and retinoblastoma hypophosphorylation occurred normally in FADDdd T cells, cyclin E expression and cyclin-dependent kinase 2 activation were markedly impaired in FADDdd T cells. These results demonstrate that a FADD/caspase-8-signaling axis promotes T cell cycle progression and sustained S6K activity.


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4 Abbreviations used in this paper: FADD, Fas-associated death domain protein; DD, death domain protein; FADDdd, dominant-negative form of FADD; v-FLIP, viral FLIP; S6K, S6 kinase; CDK, cyclin-dependent kinase; Rap, rapamycin; mTOR, mammalian target of Rap; Rb, retinoblastoma; Rosc, roscovitine; ChIP, chromatin immunoprecipitation; 7AAD, 7-aminoactinomycin D; SGK, serum and glucocorticoid-regulated kinase.

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between IL-2 signaling and death receptor signaling for the regulation of T cell homeostasis (26, 27).

As previously reported, FADDdd-expressing T cells had dramatic defects in clonal expansion to anti-CD3 and exogenously supplied IL-2, despite normal STAT5 phosphorylation following IL-2R stimulation (21). Similar defects as well as normal STAT5 phosphorylation have also been seen in transgenic mice expressing the viral protein MC159-viral FLIP (v-FLIP), which blocks activation of caspase-8 in the Fas-signaling complex (28). A second pathway activated by the IL-2R involves the recruitment of the Src homology 2-containing adaptor SHC, and the consequent induction of PI3K (25). As assessed by anti-phospho-Akt immunoblotting, PI3K activity in response to IL-2 was overtly normal in FADDdd T cells. However, using a proteomics approach with an Ab generated to detect the phosphorylated substrates of Akt and other members of the serum and glucocorticoid-regulated kinase (SGK) family, we found that sustained activation of S6 kinase 1 (S6K1) and phosphorylation of ribosomal S6 protein were defective in FADDdd-expressing T cells following stimulation with IL-2. In accord with this, FADDdd T cells completely failed to phosphorylate S6 on serines 240 and 244, sites found to be phosphorylated late during T cell activation. Induction of caspase activation has been reported to be important for T cell expansion following antigenic stimulation (29–31). Strikingly, a similar defect in the phosphorylation of S6 was observed in v-FLIP-transgenic T cells and in T cells bearing a conditional deletion of caspase-8 (casp8−/−). This failure to phosphorylate S6 in FADDdd T cells was observed even with acute stimulation of the IL-2R. Because blockade of cell cycle progression in late G1 stage with the cyclin-dependent kinase (CDK) inhibitor roscovitine (Rosc) also prevented S6 phosphorylation, our results demonstrate that the failure to phosphorylate S6 in FADDdd, v-FLIP, and casp8−/− T cells is likely a consequence of defective cell cycle progression during the G1/S transition. Thus, in addition to accepted roles in promoting apoptosis following death receptor binding, these results define a novel signaling paradigm in which sustained S6K activation depends upon FADD, cellular FLIP, and caspase-8.

Materials and Methods
Mice

1017-FADDdd-transgenic mice (Tg(Lck-FADDdd)10/10) previously described (16) were maintained on the C57BL/6 background. Mice bearing a floxed allele of caspase-8 (32) and containing 10% FCS and then activated under the indicated conditions. At given time points, cells were collected and

Flow cytometry analysis
A total of 1 × 10⁶ spleen and lymph node cells were labeled with 2.5 μM CFSE at room temperature for 8 min, washed three times with RPMI 1640 containing 10% FCS and then activated under the indicated conditions. At various times after noted stimulation, cells were stained twice with 1× PBS containing with allophycocyanin-CD4 (eBioscience), PerCP-Cy5.5 (BD Pharmingen), and PE-conjugated recombinant annexin-V (Caltag Laboratories). Cells were harvested using a FACSCalibur flow cytometer (BD Biosciences) and the amount of DNA was quantitated by flow cytometry.
CDK2 kinase assay

Wild-type, FADDdd, and casp8−/− T cells were activated for 36 h using anti-CD3 (200 ng/ml) and IL-2 (50 U/ml) in presence or absence of Rosc. Following activation, CDK2 was immunoprecipitated overnight, then subjected to an in vitro kinase assay with [γ-32P]ATP and histone H1 (Cell Signaling Technology) as substrate. The reaction was resolved by SDS-PAGE, and kinase activity was assessed by autoradiography. As a control for loading, the gel was stained with Coomassie to detect total histone H1.

Results

Defective IL-2-induced S6K activity in FADDdd T cells

We have found that FADDdd T cells fail to undergo productive proliferation in response to anti-CD3 plus anti-CD28 (21, 24), possibly due to a failure to respond to endogenously produced IL-2. Addition of exogenous rIL-2 failed to restore proliferation (Fig. 1A). Whereas wild-type T cells effectively entered into S phase, a diminished proportion of FADDdd T cells were observed to have taken up BrDU at 24 and 48 h. Instead, many of these cells had an apoptotic, subdiploid morphology, as assessed by 7AAD staining (Table I). These results suggest a defect in signaling in response to IL-2R ligation. Previously, we demonstrated that STAT5 tyrosine phosphorylation occurs normally, indicating that the IL-2R complex is overtly functional in FADDdd T cells (21). A parallel and essential signal transduction pathway activated by IL-2R stimulation involves the recruitment of SHC to the receptor, and subsequent activation of PI3K (25, 26, 34). PI3K promotes the membrane recruitment of protein kinase B/Akt, which itself plays a central role in mediating critical cellular responses including cell growth and survival (34). To assess the activation status of the PI3K pathway in mitogenically stimulated FADDdd T cells, purified wild-type or FADDdd cells were cultured for the indicated times with plate bound anti-CD3 and exogenous IL-2. Western blots were prepared and serially probed with Abs for phospho-Akt (Ser473) and compared with loading using an Ab specific for total Akt. Despite difficulty observing induction of Akt phosphorylation over basal levels, no consistent differences were observed in the level of Akt phosphorylation in FADDdd T cells when compared with wild-type cells (Fig. 1B) (the slightly elevated level of phospho-Akt observed at 90 m for FADDdd T cells was not consistent). Furthermore, the PI3K inhibitor LY294002 (LY) eliminated both induced and basal phospho-Akt to the same extent in wild-type and FADDdd T cells, demonstrating that Akt dephosphorylation occurs at a normal rate in FADDdd T cells. By comparison, treatment with the mTOR inhibitor Rap, an agent not thought to interfere with PI3K signaling, failed to inhibit Akt phosphorylation. These results demonstrate that PI3K signaling induced via the TCR complex and/or IL-2R is not altered by FADDdd expression in T cells, and are concordant with previously published results demonstrating normal Akt Ser473 phosphorylation following long-term stimulation of FADDdd-expressing T cells (17).

Although we observed normal Akt phosphorylation following IL-2R stimulation, we considered the possibility that the signaling defect imposed by FADDdd may exist further downstream of Akt activation. Additionally, phosphorylation of Ser473 might not serve as a direct readout of Akt activity. To address these considerations, we used a proteomics-based strategy to characterize differential phosphorylation of putative Akt substrates between FADDdd and wild-type T cell lysates (35, 36). Western blots from FADDdd and wild-type T cell cultures were probed with an Ab developed to detect the phosphorylated substrates of Akt (35, 37), an Ab that has been demonstrated to efficiently bind to phospho-proteins containing the Akt substrate recognition motif RxRx(R/T)(Y/S)(38). With this reagent, it is possible to classify, at least in theory, all of the

**Table I. Cell cycle progression analyzed by continuous BrdU labeling of FADDdd vs wild-type (WT) cells**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Time (h)</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
<th>Subdiploid</th>
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<td>86</td>
<td>1</td>
<td>0</td>
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<td>21</td>
<td>44</td>
<td>20</td>
<td>13</td>
</tr>
<tr>
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<td>0</td>
<td>75</td>
<td>1</td>
<td>0</td>
<td>23</td>
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<td></td>
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<td></td>
<td>48</td>
<td>37</td>
<td>16</td>
<td>6</td>
<td>40</td>
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* Percentages shown correspond to the gates labeled in Fig. 1A for T cells stimulated with anti-CD3 plus IL-2 for the indicated time.
FIGURE 2. Defective phosphorylation of ribosomal protein S6 in mitogenically stimulated FADDdd T cells. A, Failure to maintain phosphorylation of ribosomal protein S6 at serines 235 and 236 in stimulated FADDdd T cells. Immunoblot of phosphorylated (Ser235/236) in activated wild-type and FADDdd T cells following stimulation for the indicated times with anti-CD3 and human rIL-2 (50 U/ml). To block P13K or mTOR signaling, LY or Rap was added, respectively. Blots were stripped and reprobed with anti-beta-actin to control for protein loading. B, Defective S6 phosphorylation at serines 240 and 244 in activated FADDdd T cells. Western blot of activated wild-type and FADDdd T cells following 18 h of stimulation with anti-CD3 plus IL-2, in the absence or presence of LY, probed with anti-phospho-S6 (240/244); lysates were stripped and reprobed with anti-beta-actin to control for loading. LY was used at 10 μM, and Rap was used at 10 μM.

potential substrates of Akt and related kinases under various stimulation conditions. The results of this experiment revealed a roughly 32-kDa Akt- and phospho-substrate-specific (AKTSub) band that was present in mitogenically stimulated wild-type cells (Fig. 1C). The induction of this species was dramatically reduced in FADDdd T cells. Anti-CD3 or IL-2 alone were not sufficient to induce this band, suggesting that activation of both TCR- and IL-2R-linked signaling pathways is necessary to optimally elicit the appearance of this putative phosphoprotein. Pretreatment of cells with LY prevented the appearance of the 32-kDa AKTSub species, demonstrating that P13K activity is required for its induction. Although many other bands were similarly detected with this Ab, we did not observe a consistent dependence on FADD, PI3K or mitogenic signaling for their appearance at this late time after activation (data not shown). Furthermore, because this band was not enhanced in FADDdd lysates after treatment of the cells with okadaic acid or calyculin, its lack of appearance in these cells is not likely to be due to hyperactive serine/threonine-directed phosphatase activity (data not shown).

Previously, we found that the AKTSub Ab detected phosphorylated ribosomal S6 protein in BCR-Abl-transformed chronic myelogenous leukemia tumors (39), yielding a band of roughly 30 kDa. Additionally, S6Ks share motif specificity with other SGK family kinases, including Akt (38). Thus, we hypothesized that the band detected by the AKTSub Ab may be S6 rather than a direct Akt substrate. To determine whether S6 phosphorylation was defective in FADDdd T cells, purified T cells were activated with anti-CD3 plus IL-2 for various times, lysed, and subjected to Western blotting with anti-phospho-S6. Treatment of both wild-type and FADDdd T cells promoted weak S6 phosphorylation at serines 235 and 236 as early as 30 min following stimulation (Fig. 2A). Pretreatment of cells with LY or Rap reduced this level of phosphorylation to below basal levels, as expected. With time, both total S6 and phospho-S6 (Ser235/236) accumulated, with highest levels observed after 18 h of stimulation. Although phosphorylation of S6 at serines 235 and 236 was observed in FADDdd T cells at early times, these cells possessed substantially reduced levels of phospho-S6 (Ser235/236) at 18 h.

S6 is sequentially phosphorylated, with initial phosphorylation detected at serines 235 and 236, followed by subsequent phosphorylation at serines 240, 244, and 247 (40). Phosphorylation of S6 at positions 240 and 244 was not observed at 5 h. After 18 h of treatment with anti-CD3 plus IL-2, substantial phosphorylation of S6 on these serines was observed. These data demonstrate that the sustained activity of S6Ks is necessary to promote full phosphorylation of this riboprotein following mitogenic stimulation of primary, naive T cells (Fig. 2B). Consistent with the hypothesis that phosphorylation at serines 240 and 244 requires sustained S6K activity, FADDdd T cells failed to promote phosphorylation of S6 at these positions, even after 18 h mitogenic stimulation. Again, incubation of activated FADDdd T cells in phosphatase inhibitors did not impact S6 phosphorylation (data not shown). Thus, while FADDdd T cells do not possess a general deficit in phosphorylation or hyperactive dephosphorylation of S6, they fail to sequentially phosphorylate serine residues necessary for maximal S6 function.

Defective S6K1 phosphorylation in FADDdd-transgenic T cells

Because a defect in S6 phosphorylation in FADDdd T cells was observed, the phosphorylation status of S6K1 at position Thr389 was measured; phosphorylation at this site is essential for the activity of the kinase (41). Wild-type and FADDdd T cells were activated for 2, 6, or 18 h in the absence or presence of LY and lysates blotted and probed with an Ab to detect phosphorylated S6K1 at Thr389 (Fig. 3A). Although early phosphorylation of S6K1

FIGURE 3. Defective sustained S6K1 phosphorylation, but normal mTOR function in FADDdd T cells. A, Lack of sustained S6K1 phosphorylation in FADDdd T cells. Cells were stimulated with anti-CD3 (50 ng/ml) and IL-2 (50 U/ml) for the indicated times in the absence or presence of LY magnetically purified, and then used for Western blotting with anti-phospho-S6K1 (Thr389) or total S6K1 Abs. B, Normal pattern of 4E-BP1 phosphorylation in FADDdd T cells. Cells were stimulated for the indicated times with and without LY. Immunoblots were probed with anti-4E-BP1; lower mobility phosphorylated isoforms (β and γ) detected by Western blotting are indicated. To control for loading, the blot was stripped and reprobed with anti-beta-actin. C, Normal pattern of elf4E phosphorylation in mitogenically stimulated FADDdd T cells. Western blots of cells treated as in A were probed with Abs to phospho-elf4E (Ser37/38), or anti-beta-actin to control for protein loading.
was normal in FADDdd T cells, the level of S6K1 phosphorylation was diminished at 18 h in FADDdd, but not wild-type cells. Together with results using anti-phospho-S6-specific Abs, this finding suggests that FADD function is required for the long-term maintenance of S6K1 activity. To determine whether the failure to maintain sustained S6K1 phosphorylation was due to a general deficit in mTOR signaling, the phosphorylation status of 4E-BP1 was evaluated because this inhibitor of eIF4E is phosphorylated in an mTOR-dependent manner. With stimulation, there was a shift in the mobility of 4E-BP1 to slower migrating isoforms in both wild-type and FADDdd T cells (Fig. 3B). Because treatment of FADDdd T cells with Rap blocked residual proliferation (data not shown), these results suggest that mTOR signaling was unimpaired in FADDdd T cells after 18 h of stimulation. A parallel signaling pathway crucial to growth-factor induced translation involves the phosphorylation of the eukaryotic cap-binding translation initiation factor elf4E at serine 209 via a p38/Erk→Mnk1 pathway (42, 43). Examination of this pathway revealed normal phosphorylation of elf4E in FADDdd T cells following stimulation with anti-CD3 and IL-2 (Fig. 3C). This phosphorylation was not blocked by LY or Rap, consistent with previous results demonstrating that elf4E phosphorylation at Ser209 depends on p38 and Mnk1, and occurs independently of P38K or mTOR signaling (42). Taken together, these results demonstrate that FADDdd T cells fail to maintain S6K1 phosphorylation following mitogenic stimulation, but that 4E-BP1- and elf4E-signaling pathways are intact.

**FADDdd T cells have a diminished CDK2 activity during entry into S phase**

Due to the dramatic decrease in cell cycle progression and growth observed in FADDdd T cells above, we wished to evaluate the status of early cell cycle regulatory proteins. In particular, we focused our attention on those proteins known to be associated with progression from G0 to S phase because FADDdd T cells exhibit reduced progression through this stage of the cell cycle (24). D-type cyclins promote activation of CDK4 and 6, kinases that induce exit from G0 (44). Consistent with previous results (45), activation of purified T cells with plate-bound anti-CD3 and IL-2 promoted increased expression of cyclin D3 after 18 h of stimulation (Fig. 4A). Increased expression of this early G1 cyclin was not inhibited by FADDdd expression. No defect was observed in p27Kip1 down-modulation, further suggesting that the early G0→G1 transition occurred normally in FADDdd-expressing cells (data not shown). Rap treatment substantially inhibited the expression of cyclin D3, consistent with a recent report demonstrating that cyclin D3 translation requires mTOR activity (46). Similarly, treatment of wild-type T cells with anti-CD3 and IL-2 promoted up-regulation of cyclin E expression, although we could not detect the presence of this G1/S cyclin until after 18 h of mitogenic stimulation. We did not detect the presence of cyclin E expression in FADDdd T cells, even after 30 h of mitogenic stimulation. As was the case for cyclin D3, cyclin E expression was also reduced by pretreatment with Rap. The CDK2/cdk2 inhibitor Rosc inhibited the up-regulation of cyclin E, while only having a moderate effect on cyclin D3 induction, the latter result consistent with previous reports in which the increased level of expression of cyclin E depends on CDK2 activity (47). Thus, based on these studies, we hypothesize that FADDdd T cells progress normally through early G1 phase, but have a defect in the CDK2-dependent transition to S phase.

We also wished to determine the phosphorylation status of retinoblastoma (Rb), a key cell cycle inhibitory protein known to be phosphorylated on multiple residues by G1 cyclin/CDK complexes (47). Rb was phosphorylated at serines 807 and 811 as early as 6 h following stimulation with anti-CD3 and IL-2, with maximal phosphorylation observed after 24 h (Fig. 4B, upper panel). Phosphorylation at these sites was similar in FADDdd T cells following activation, a finding consistent with a recent report demonstrating that CDK4/6: cyclin D complexes promote phosphorylation at these sites (48). An additional site phosphorylated by CDK4/6: cyclin D complexes is serine 780. However, this site was found to be constitutively phosphorylated in untreated wild-type and FADDdd T cells, regardless of activation status (Fig. 4B, middle panel). During transition from early G1 to late G1, cyclin E levels increase, promoting activation of CDK2. Elevated levels of active cyclin E: CDK2 complexes promote hyperphosphorylation of Rb at many additional sites (49). Although difficult to visualize, we observed a putative hyperphosphorylated Rb band in wild-type, but not FADDdd T cells, following activation with anti-CD3 plus IL-2 for 24 h (Fig. 4B, lower panel). Treatment with the CDK2/cdk2 inhibitor Rosc at 10 μM failed to inhibit phosphorylation at serines 780, 807, and 811. However, like FADDdd expression, Rosc prevented the appearance of the hyperphosphorylated Rb band. These results suggest that FADDdd T cells may have a defect in the induction of CDK2 activity during the G1/S transition. To test this, wild-type and FADDdd T cells were mitogenically stimulated for 24 h as above, in the absence and presence of Rosc. Lysates were immunoprecipitated with anti-CDK2, followed by in vitro kinase
Figure 5. Sustained S6 phosphorylation depends upon IL-2-dependent cell cycle progression. A. Blockade of IL-2-driven S6 phosphorylation by Rosc. Cells were preincubated with LY, Rosc, Rap, or left untreated, followed by anti-CD3 plus IL-2 culture for 24 h. Lysates were probed with anti-phospho-S6 (S240/S244). The blot was stripped and reprobed with anti-Actin. B, Blockade of S6K1 phosphorylation by Rosc. Cells were stimulated for the indicated times in the absence or presence of Rosc, followed by immunoblotting as above. C. Acute IL-2 signaling leads to S6 phosphorylation. Cells were stimulated with anti-CD3 (50 ng/ml) in the absence or presence of anti-mouse IL-2 (5 μg/ml) to block autocrine signaling. Human rIL-2 (100 U/ml) was added for the indicated times, or provided continuously for the duration of the culture (“cont”), followed by lysis and anti-phospho-S6 (S240/S244) staining. D. Blockade of acute and long-term IL-2 induced S6 phosphorylation by Rosc. Cells were incubated as in C, except that some cells were preincubated with Rosc before activation by plate-bound anti-CD3. Blot was stripped and reprobed with anti-phospho-ERK1/2.

Assays using histone H1 as substrate. Whereas wild-type cells possessed a high level of CDK2 activity, and this activity was blocked by Rosc, FADDdd T cells lacked appreciable CDK2 activity (Fig. 4C). Taken together, these results demonstrate that while early G1 cell cycle progression occurs normally in FADDdd T cells, later stages dependent upon cyclin E:CDK2 are defective in these mutant cells.

Sustained S6 phosphorylation depends upon IL-2-driven CDK2 activity

To determine whether the defect in sustained S6 phosphorylation might be a consequence of diminished CDK2 activity, we evaluated the potential that agents blocking cell cycle progression might reduce S6 phosphorylation. Treatment of T cells with anti-CD3 plus IL-2 promoted S6 phosphorylation at S240/S244, as observed previously. As expected, pretreatment with LY or Rap blocked this. Pretreatment with Rosc prevented S-phase entry, as assessed by BrdU analysis (data not shown), and also inhibited sustained S6 phosphorylation (Fig. 5A). Rosc pretreatment also blocked sustained S6K1 phosphorylation (Fig. 5B). These results suggest that sustained S6 phosphorylation induced by IL-2 requires CDK2 activity. To determine whether IL-2R signaling is responsible for sustained S6 phosphorylation, T cells were preincubated with anti-murine IL-2 to block autocrine signaling through the IL-2R via endogenously produced cytokines, followed by 24 h stimulation with plate-bound anti-CD3. This treatment greatly reduced the number of cells entering into S phase at 24 h (data not shown). In comparison to cells receiving continuous IL-2 during the 24 h culture, cells pretreated with anti-IL-2 had diminished S6 phosphorylation (Fig. 5C). Treatment with human rIL-2 (which is not blocked by the anti-mouse IL-2 Ab used in the assay) reversed the S6 phosphorylation defect, indicating that S6 phosphorylation on serines 240 and 244 is dependent on IL-2R signaling. Under all of these conditions, S6 was only weakly phosphorylated in FADDdd T cells (data not shown). Rosc blocked S6 phosphorylation in cells receiving both short- and long-term IL-2 treatment after 18 h anti-CD3 stimulation, suggesting that sustained S6 phosphorylation induced via IL-2R signaling depends upon cell cycle transit. Because ERK phosphorylation induced by short-term IL-2 treatment of pre-activated T cells was not inhibited by Rosc, it is unlikely that Rosc blocked global IL-2R signaling. These results demonstrate that the sustained S6 phosphorylation in T cells that is blocked in FADDdd T cells depends upon IL-2R signaling, and that this process likely requires active cell cycle entry.

Caspase-8 activity is necessary for efficient cell cycle progression and sustained S6 phosphorylation in T cells

In addition to FADD, caspase activation has also been shown to be essential for T cell proliferation (29, 30, 50, 51). To determine whether a deficiency in caspase-8 activity might also impact S6 phosphorylation, we made use of two mutant lines of mice. First, we tested mice expressing a T cell-specific transgene encoding v-FLIP, a viral inhibitor of caspase-8. These mice have been previously shown to bear T cells with deficiencies in proliferation similar to those observed in FADDdd mice (28). STAT5 activity was assessed using a ChIP assay with the oncostatin M promoter, a target of STAT5 activity (52). Because no defect in binding to the oncostatin M promoter was observed (Fig. 6A), we conclude that STAT5 activity downstream of the IL-2R is unimpeded by the v-FLIP transgene. v-FLIP and wild-type T cells were preactivated with anti-CD3 plus anti-CD28, followed by short-term stimulation with IL-2 to assess STAT5 phosphorylation. As predicted by the normal STAT5 binding to the oncostatin M promoter, no obvious defects were observed in STAT5 phosphorylation in v-FLIP-transgenic T cells (Fig. 6B, upper panel). We also assessed the phosphorylation status of S6K1 and observed a modest but reproducible defect in S6K1 phosphorylation relative to total S6K1 (Fig. 6B, lower panel). When stimulated continuously with anti-CD3 plus IL-2 for 24 h, v-FLIP T cells failed to accumulate phosphorylation on serines 240 and 244, similar to results obtained with FADDdd T cells (Fig. 6C). These results suggest that v-FLIP interferes with optimal S6K1 phosphorylation and activity following IL-2R ligation.

To determine whether caspase-8 itself is necessary for sustained S6 phosphorylation, we tested caspase-8-deficient T cells produced by crossing mice bearing conditionally mutant caspase-8 (Casp8-Flox) alleles (32) with mice expressing the Cre recombinase under a T cell-specific promoter (CD4Cre). First, we assessed proliferation and cell cycle using CFSE labeling and BrdU/7AAD analysis. As observed for FADDdd T cells, casp8null T cells had a reduced level of proliferation and survival, as assessed by CFSE dilution analysis (Fig. 7A). This diminished proliferation was primarily a consequence of decreased survival and was most obvious in the
population, consistent with our previous results using FADDdd expressing mice and those reported for another line of mice bearing a conditional mutation in caspase-8 (21, 50). To determine whether casp8−/− T cells also possess a defect in cell cycle progression, we stimulated cells for 24 and 48 h with anti-CD3 plus IL-2. Wild-type and casp8−/− T cells were labeled with CFSE before culture for 3 days in the presence of anti-CD3 (50 ng/well) plus IL-2 (50 U/ml). A, Cell cycle analysis of magnetically purified casp8−/− T cells following stimulation with anti-CD3 plus IL-2. To assess DNA synthesis, BrdU was added during the entire culture. After the indicated times, cells were permeabilized, then stained with FITC-conjugated anti-BrdU Abs and 7AAD. C, Diminished S6 phosphorylation in casp8−/− T cells following mitogenic stimulation. Cells were stimulated for the indicated times using plate-bound anti-CD3 plus IL-2. Where indicated, cells were preincubated with LY. Lysates were then subjected to immunoblot analysis using anti-phospho-S6 (S235/S236), anti-phospho-S6 (S240/S244) and anti-β-actin Abs. D, Diminished CDK2 activity in activated casp8−/− T cells. Casp8−/− or wild-type T cells were activated with anti-CD3 plus IL-2 for 24 h, followed by lysis and immunoprecipitation with anti-CDK2. Immunoprecipitates were analyzed for histone H1 phosphorylation activity, without or with added Rosc, as in Fig. 4C. E, Potential model for FADD/caspase-8-dependent S6K1 signaling. FADD and caspase-8 promote inactivation of cell cycle inhibitor that interferes with CDK2/cyclin E ("E") function. This prevents CDK2/cyclin E-dependent phosphorylation of S6K1, which along with mTOR-dependent phosphorylation, leads to full S6K1 activity.

CD8+ population, consistent with our previous results using FADDdd expressing mice and those reported for another line of mice bearing a conditional mutation in caspase-8 (21, 50). To determine
Perhaps due to diminished cell cycle entry and decreased CDK2 activity, T cells with deficiencies in FADD and caspase-8 signaling also failed to maintain S6 phosphorylation at normal levels, especially on serines 240 and 244.

Discussion

We have established that, in addition to promoting death-receptor dependent apoptotic pathways, FADD and caspase-8 play an essential role in maintaining S6K1 activity during T cell cycle progression. We have found that prolonged T cell activation is essential for complete serial phosphorylation of ribosomal protein S6, and potentially other S6K1 substrates. In accord with this defective S6K1 activity, we have found that naive T cells failed to efficiently enter S phase, and possessed defective CDK2 activity, cyclin E induction, and Rb hyperphosphorylation (Fig. 4). Although FADDdd T cells failed to maintain S6K1 and S6 phosphorylation during late G1 phase, we found that mTOR function was apparently normal because phosphorylation of 4E-BP1 was unaltered in the mutant cells. Because FADDdd T cells had diminished CDK2 activity, and because a specific inhibitor of CDK2 reduced S6 phosphorylation on S240/S244, we propose that sustained S6 phosphorylation depends on active cell cycle progression induced by IL-2. Our data also demonstrate similar defects in cell cycle progression and S6K1 activity in T cells derived from v-FLIP-transgenic and caspase-8 conditionally null mice, suggesting that FADD and caspase-8 are both essential for optimal cell cycle progression and survival following mitogenic stimulation. Taken together, these findings suggest that FADD, caspase-8 and cellular FLIP operate together to promote efficient cell cycle progression in mitogenically stimulated T cells.

The means by which FADD and caspase-8 participate in S6K1 function is currently unclear. Because S6K1 signaling was normal immediately following TCR cross-linking (Fig. 3), our results demonstrate that the effect of the FADDdd transgene is not likely directed toward mTOR or other inputs into this signaling pathway (53). Rather, we consider it likely that FADD and caspase-8 play a distal role in maintaining S6K1 activity, likely during the late G1 phase of cell cycle. This defect is temporally distinct from the reduced NF-kB activation seen in human caspase-8-deficient T cells, which occurs within minutes of TCR triggering (23, 54). Our data demonstrate that in primary murine T cells, the long-term activation of S6K1 is required for complete phosphorylation of ribosomal protein S6 (Fig. 3). S6 possesses five distinct serine residues that are known to be sequentially phosphorylated by S6K1 and S6K2 (55). In response to mitogenic stimulation with anti-CD3 and IL-2, serines 235 and 236 became phosphorylated within 90 min, whereas phosphorylation at serines 240 and 244 was not observed until much later (Fig. 2). Recently, it was shown that S6K1\textsuperscript{-/−}/S6K2\textsuperscript{-/−} embryonic fibroblasts retained the capacity to phosphorylate S6 on serines 235 and 236, whereas phosphorylation at serines 240 and 244 was completely blocked (56). Additionally, although phosphorylation of S6 on serines 235 and 236 is highly dependent upon p90/RSK and Ras/ERK signaling, 240 and 244 are exclusively phosphorylated by S6K1 and S6K2 (57). These results demonstrate that although phosphorylation of S6 at serines 235 and 236 may occur via S6K1/S6K2-independent means, complete phosphorylation of S6 depends on the function of these kinases. Our results are consistent with the notion that defective phosphorylation of S6K1 at Thr\textsuperscript{389}, a site essential for the activity of this kinase, completely abrogates serial S6 phosphorylation at sites 240 and 244.

As described here, phosphorylation of S6 at S240/S244 was blocked by the CDK2 inhibitor Rosc. This result suggests a potential feedback mechanism whereby induction of CDK2 before S-phase entry leads to enhanced ribosome biogenesis and macromolecular synthesis required for later stages in cell cycle. Indeed, recent evidence has demonstrated that both CDK1 and CDK5 have the potential to phosphorylate S6K1 directly. In the case of CDK5, phosphorylation of S6K1 on Thr\textsuperscript{411} has been demonstrated to be required for its phosphorylation and activation by mTOR, suggesting that such a feedback loop between CDK2 and S6K1 may exist in cycling T cells (58, 59). That S6 is itself important for T cell proliferation has been established in mice expressing one defective allele for S6 (60). In these mice, T cells bear a similar defect in proliferation as we have observed in FADDdd and casp8\textsuperscript{-/−} T cells. It is notable that S6\textsuperscript{+/−} T cells have diminished S-phase entry and survival following mitogenic stimulation, and this is most strikingly evident in CD8\textsuperscript{+} T cells. This selective defect may be a consequence of the higher rate of cell cycle progression observed in CD8\textsuperscript{+} T cells.

One explanation may be that FADD and caspase-8 modulate the activity of a key signaling molecule for late G1 progression, and that this step maintains or enhances the active state of S6K1, likely via active CDK2. S6K1 then facilitates cell growth during late G1 and promotes subsequent steps of cell cycle. For example, both PI3K and S6K1 are essential for modulating E2F in response to IL-2, an event necessary for promoting S-phase entry (61–65). An alternative explanation that may account for the results reported here is that FADD and caspase-8 in some way govern the signaling cascade that leads from the IL-2R to S6 phosphorylation. However, it is unlikely that FADDdd and v-FLIP-transgenic T cells possess a global defect in IL-2R function, because we observed normal STAT5 and Akt phosphorylation (Ref. 21 and Figs. 1, 3, and 6). Rather, the defect appears to be selective, at a level between mTOR and S6K1 (Fig. 7E). Thus, an alternative scenario is that the observed defects in CDK2 activity may be a consequence rather than a cause for diminished S6K1 function. Recent studies have uncovered an interaction between the anti-apoptotic factor survivin, mTOR, and aurora B kinase, an interaction essential for T cell proliferation and survival (66). Death effector domain-containing protein (DEDD), a protein sharing a homologous death effector domain with FADD, also has been shown to control cell cycle via the CDK1/cyclin B1 complex (67). Our own recent work suggests that FADDdd and casp8\textsuperscript{-/−} T cells possess a hyperautophagic phenotype (B. Bell et al., manuscript in preparation). Because mTOR and S6Ks directly impact autophagy (68), our studies here highlight an additional signaling paradigm in which the mTOR pathway is subject to regulation by an apoptotic-signaling intermediate.

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Disclosures

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