Expression of a truncated form of the death receptor adaptor FADD (C-FADD) as a transgene in mice blocks T cell proliferation. Here we provide evidence that the C-terminal phosphorylation site Ser$^{194}$ in C-FADD affects the cell cycle in nonlymphoid cells as well. High expression of wild type C-FADD but not C-FADD with a S194A point mutation arrested the nontumor cell line MCF10A in G$_2$/M but not the tumor cell line MCF7. BJAB as well as MCF10A cells expressing moderate levels of C-FADD with a S194E mutation mimicking phosphorylated C-FADD were more susceptible to a Taxol®-induced G$_2$/M arrest than cells expressing C-FADD S194A suggesting that C-FADD S194E lowers the threshold for G$_2$/M arrest. Our data suggest that C-FADD may affect apoptosis sensitivity of cells by interfering with cell cycle progression and not only by binding to death receptors.

FADD is a death domain (DD) and death effector domain (DED) containing adaptor protein essential for recruitment of the initiator caspases 8 and 10 to activated death receptors during apoptosis (1). Overexpression of the C-terminal half of FADD (C-FADD/DED-DN), which contains the DD but lacks the DED, has been shown to have a dominant interfering effect, protecting against death receptor-mediated apoptosis by competing with endogenous FADD for binding to death receptors (2).

FADD$^{-/-}$ mice die in utero, which suggests a role for FADD in embryonic development (3, 4). Furthermore, FADD may also be a necessary component in T cell development and activation. Thymocytes and peripheral T cells from transgenic mice expressing C-FADD under the control of the proximal lek promoter exhibit a defect in activation-induced proliferation (5). Similarly, FADD$^{-/-}$ T cells in chimeric mice of the RAG-1$^{-/-}$ background, which themselves lack T cells, also show impaired proliferation following activation (3) resulting in a block in early T cell development (6, 7) suggesting that in resting T cells FADD is important for reentry into the cell cycle.

We recently showed that FADD and C-FADD are selectively phosphorylated at Ser$^{194}$ (8). Highly phosphorylated FADD was found in cells in G$_2$/M, whereas FADD phosphorylation was low in G$_1$/S. Recently, the ability of FADD to promote cell cycle progression of T cells was found to depend on the phosphorylation of this site (9). We now provide evidence that the Ser$^{194}$ phosphorylation site is responsible for the ability of C-FADD to interfere with the cell cycle. Our data suggest that C-FADD may affect cell growth through the activity of a third domain located in the 35 C-terminal amino acids distinct from the DED and the D.

### EXPERIMENTAL PROCEDURES

**Cell Lines and Reagents—**Culturing of BJAB cells was described previously (8). BJAB stable transfectants were generated by transfection of C-FADD pcDNA3 plasmids as described before (8). BJAB and MCF7-Fas stable transfectants were further supplemented with 1 or 0.25 mg/ml G418, respectively. MCF7 cells were cultured in Dulbecco’s modified Eagle’s medium, 10% FCS, 100 mM HEPES, and 50 µg/ml gentamycin. MCF10A and derivative cells were cultured in Dulbecco’s modified Eagle’s medium-F-12 supplemented with 5% FCS, 0.5 µg/ml hydrocortisone, 20 ng/ml human recombinant epidermal growth factor, 10 µg/ml insulin, and 10 µg/ml gentamycin. MCF10A-Myc cells were kept in selection with 0.2 mg/ml G418. MCF10A-Myc cells were a gift from Dr. Conzen (University of Chicago) and described before (11). Taxol (Paclitaxel) was obtained from Sigma (catalog number T-7402).

Cells were arrested with low doses of Taxol that do not induce apoptosis: 50 nm Taxol for BJAB and 500 nm for MCF10A-Myc cells.

Adenoviral Constructs and Transduction—GFP and C-FADD expressing adenovirus-5 constructs were described before (19). Generation of the S194A point mutant has also been described previously (8). AdV-5A vector as well as the AdV-GFP control vector were generated using the two-cosmid system as described before (20). In brief, the C-FADD gene expression unit with a cytomegalovirus promoter was cloned into the pLEP plasmid, using two cloning primers (AdV-C-FADD HindIII CCCAAACTTGGGATGGACACATACCGCTAC and AdV-C-F-ADD NoI ATAGTTTACGCGCCCGGATTTATTTCAAGGAGCCGGTCG- GAGGT) and ligated into the pREB plasmid, containing the E1-deleted adenovirus type 2 genome. Recombinant Ad$^+$ adenoviruses were generated in the E1-complemented helper cell line 293A. High titer stock solutions of adenoviruses were obtained by repeated amplifications in 293A, cesium chloride purification, and quantification by measuring absorbance at A$_{600}$.

For infection of cells with adenovirus, MCF7 or MCF10A cells were cultured to ~70% confluence before transduction. The cells were incubated in 2% FCS low serum, cell type-specific medium containing recombinant adenovirus at a multiplicity of infection of 100 viral particles/cell at 37°C for 90 min, and then supplemented to 10% FCS containing medium. Transduction efficiency was determined by intracellular staining for AU1-tagged C-FADD.

Retroviral Constructs and Transduction—The C-FADD S194E point mutant was generated with the QuikChange XL mutagenesis kit (Stratagene) using complementary primers to the sequence GTGGGGCCATGGACGGATCTG (sense). To generate retroviral constructs, C-FADD wt (amino acids 80–208 of human FADD) and point mutants S194A and S194E were subcloned into the bicistronic retroviral vector MigR1 (MSCV-IRE5-GFP, as described previously (21)) by ligation into the MigR1 and EcoRI sites. High titer retroviral supernatants were generated using the 9NX-Ampho helper cell line (21). Packaging cells were cotransfected with MigR1-C-FADD vectors and a vesicular stomatitis virus G glycoprotein coding plasmid (22) using Polyfect transfection reagent (Qiagen). Following transfection, supernatants were isolated at 48 h and added to cells along with 10 µg/ml polybrene, followed by a 1-h centrifugation step at 2700 g to enhance infection. Following transduction, cells were allowed to grow and sorted 2 weeks later based on GFP positivity to obtain a homogenous population.
Phosphorylation of FADD and Cell Cycle

Intracellular Staining—For the quantification of intracellularly expressed proteins, cells were stained by a saponin permeabilization method (23). The primary antibody used was the anti-AU1 (IgG3) from Covance (Richmond, CA) and secondary fluorescein isothiocyanate-coupled goat anti-mouse IgG from Santa Cruz Biotechnology (Santa Cruz, CA).

DNA Fragmentation and Cell Cycle Analysis—For apoptosis assays cells were stimulated with 1 μg/ml anti-APO-1 plus 1 ng/ml protein A or control treated with protein A alone and incubated for 16 h at 37 °C. Anti-APO-1 antibody is an agonistic anti-CD95 antibody. For the quantification of DNA degradation or cell cycle analysis 5 × 10^6 cells were incubated with propidium iodide (8) and DNA content was measured by fluorescence-activated cell sorter analysis (BD Biosciences). Western Blotting—Immunoblots for FADD were done as described before (8) using the anti-FADD (IgG1) antibody from Transduction Laboratories (Lexington, KY).

RESULTS AND DISCUSSION

The Ser^{194} Phosphorylation Site in C-FADD Is Not Important for Its Inhibition of CD95-mediated Apoptosis—Within FADD, Ser^{194} is part of a C-terminal domain outside of the DD. We therefore postulated that this region is not involved in recruiting FADD to the activated CD95 receptor. This suggested that phosphorylation of FADD at this residue does not interfere with apoptosis signaling through CD95 and may not function in apoptosis signaling. It has been shown that C-FADD inhibits cell cycle progression or is even toxic when expressed in non-tumor cells (10), whereas it acts only as an inhibitor of CD95-mediated apoptosis in constantly cycling tumor cells (8, 10). We therefore sought a nontumor cell line that would allow us to test the effects of C-FADD and its phosphorylation on the cell cycle. MCF10A cells are spontaneously immortalized breast epithelial cells that require both growth factors and steroids in culture (11). We used adenoviruses allowing us to express C-FADD (AdV-wt), C-FADD in which we replaced the phosphorylated serine at position 194 with alanine (AdV-SA), and GFP (AdV-GFP) as control. MCF10A cells could be quantitatively infected with these viruses, and the C-FADD proteins were expressed at the expected molecular weights (Fig. 1A). To test the effect of these proteins on apoptosis we infected the mammary carcinoma cell line MCF7-Fas with these viruses (12) (Fig. 1B). Both proteins efficiently blocked CD95-mediated apoptosis confirming that all viruses transduced expression of functional C-FADD proteins.

C-FADD Arrests MCF10A Cells in G_{2}/M—We next infected MCF10A cells with AdV-wt to test for effects on cell growth/cell cycle (Fig. 2A). C-FADD protein was expressed 2 days after infection (d2). However, beginning at day 3 post-infection the morphology of the cells changed, the proliferation stopped, and the cells exhibited a rounded morphology. At day 6 most of the cells were still attached to the plates but were rounded and appeared to be arrested in the cell cycle. Subsequent analysis demonstrated that C-FADD did not induce apoptosis in these cells as judged by the lack of fragmented nuclei following staining with propidium iodide (PI), lack of caspase processing in Western blot analysis, and the lack of PI uptake by these cells (data not shown). Furthermore, this effect of C-FADD on MCF10A cells was also observed when cells were cultured in the presence of the polyspecific caspase inhibitor benzyloxy carbonyl-Val-Ala-Asp-fluoromethyl ketone (data not shown). A cell cycle analysis revealed that C-FADD-expressing cells arrested in G_{2}/M (Fig. 2A). Interestingly, consistent with what we have reported previously, phosphorylation of endogenous FADD increased at the time of the G_{2}/M arrest (Fig. 2B), indicating that expression of exogenous C-FADD can affect the phosphorylation status of endogenous FADD.

The C-FADD-induced Cell Cycle Arrest in Nontumor Cells Requires an Intact Ser^{194} Phosphorylation Site—We next tested the effects of the different adenoviral constructs on

[FIG. 1. Adenovirally expressed C-FADD proteins are functional and confer resistance to CD95 mediated apoptosis independent of Ser^{194} phosphorylation. A, intracellular staining of MCF-10A cells transduced with adenovirus expressing C-FADD wild type (wt) or mutant S194A (SA) 2 days post-infection. The broken line is the IgG1 control antibody, and the solid line is the anti-AU1 antibody recognizing the AU1 tag of C-FADD. A corresponding Western blot is also shown. C-FADD-P indicates the phosphorylated higher migrating form of C-FADD. B, MCF7-Fas cells stably expressing CD95 were transduced with control empty virus or the AdV-C-FADD constructs wt or S194A. 24 h post-infection cells were left untreated (−) or stimulated with anti-CD95 (+). The bottom panel indicates intracellular staining with anti-AU1 and is representative of C-FADD expression at time of stimulation.]

MCF10A cells and on a corresponding tumor cell line MCF7 cells. Six days after infection MCF10A cells infected with AdV-GFP continued to proliferate (Fig. 2C), whereas MCF10A cells infected with AdV-wt had again rounded and stopped growing. In contrast, cells infected with AdV-SA continued to proliferate at a rate similar to GFP expressing cells (Fig. 2C). Interestingly, MCF7 cells expressing very similar high levels of C-
FADD were not affected in their growth and not arrested in the cell cycle (Fig. 2). These data first demonstrate that an intact phosphorylation site within C-FADD is required for the C-FADD-mediated inhibition of the cell cycle and second confirm that C-FADD acts in a cell-specific fashion.

**C-FADD-SE Lowers the Threshold for G2/M Arrest in Cells Not Arrested by C-FADD**—We and others (8, 10) have shown that certain tumor cell lines while rendered resistant to CD95-mediated apoptosis were not arrested in the cell cycle by overexpression of C-FADD. Since C-FADD arrested MCF10A cells in G2/M, and we demonstrated previously that the highest phosphorylation of FADD at Ser 194 is found in G2/M, we generated BJAB cells (a Burkitt lymphoma cell line) stably expressing C-FADD with a S194E mutation to mimic phosphorylated FADD. Similar to C-FADD-wt and C-FADD-SA-expressing BJAB cells, CD95-mediated apoptosis was inhibited in the C-FADD-SE transfectants confirming that all proteins were expressed in a functional form (Fig. 3A). Although no obvious effects on the cell cycle were observed in these transfectants, we tested whether we could detect differences in the sensitivity to a reagent that induces G2/M arrest and FADD phosphorylation such as the tubulin binder Taxol. When cells were treated with a low dose of Taxol the C-FADD-SE-expressing cells arrested earlier and much more efficiently than the cells expressing the nonphosphorylatable C-FADD-SA (Fig. 3B). This result suggested that the phosphorylation of C-FADD at Ser194 could be generally relevant for the cell cycle but that cells have different thresholds of sensitivity to this effect. We therefore expected that a low expression of C-FADD-SE in MCF10A cells would also make these cells more susceptible to Taxol-induced G2/M arrest without causing a direct cell cycle arrest. To achieve lower protein expression than observed by infecting cells with adenoviruses, we switched to a retroviral system. Because MCF10A cells grow very slowly and could therefore not be efficiently infected by retroviruses (data not shown), we used MCF10A cells transfected with c-Myc. c-Myc accelerated the growth of MCF10A cells significantly without transforming them (11). Cells were infected with the different GFP-C-FADD viruses (at an infectious yield of 40%) and after 2 weeks sorted for GFP-positive cells resulting in similar expression of C-FADD (Fig. 3C). Overexpression of C-FADD did not affect expression of c-Myc (data not shown). Similar to the BJAB cells, MCF10A-Myc cells stably expressing C-FADD-SE were not directly affected in the cell cycle but were more sensitive to Taxol-induced G2/M arrest suggesting that even under conditions in which C-FADD does not directly block cell cycle progression it still has an effect on the cell cycle and this effect is dependent on its phosphorylation at Ser194 (Fig. 3D). Our data are consistent with a model in which C-FADD-SE acts synergistically with Taxol.

A previous study has shown that C-FADD only kills normal but not tumorigenic prostate epithelial cells (10). The authors had attributed this activity to the FADD DD. However, our data make it likely that the Ser194 phosphorylation site of C-FADD is an important target for the regulation of the cell cycle.
FADD was involved in this activity. Ser$_{194}$ is located in a domain C-terminal of the DD in FADD that has thus far not been well studied. However, C-FADD has been widely used to test whether a form of apoptosis is dependent on activation of death receptors. Our data provide an explanation for the growing number of cases in which C-FADD has been shown to inhibit cell death where no involvement of death receptors/ligands could be shown (13–15). It is conceivable that in these cases C-FADD affected the cell cycle, which may have rendered them more resistant to apoptosis. Indeed, a previous report characterizing the MCF10A-Myc cells found that apoptosis induced by serum withdrawal was substantially blocked by overexpression of C-FADD, and no evidence for death receptor involvement could be found (11).

MCF10A cells in our study were arrested in G$_0$/M by C-FADD. In contrast, resting cells overexpressing C-FADD were shown to be arrested in G$_0$ or G$_1$ (16). An analysis of FADD$^{-/-}$ T cells showed that a fraction of T cells in these mice showed a 10-fold increase of cells in S and the G$_2$/M phase over wild type levels (17). It is not clear at present how the lack of FADD or overexpression of C-FADD interferes with the cell cycle in different cells. An analysis has excluded a role for the following pathways in T cells: activation of NF-$\kappa$B, mobilization of intracellular calcium, p38, and mitogen-activated protein kinases (18). The kinase that phosphorylates FADD at Ser$_{194}$ therefore likely acts in parallel or downstream of all these pathways. It has been reported that upon overexpression of C-FADD several cell cycle proteins were deregulated suggesting FADD may directly or indirectly interfere with the cell cycle machinery (18). Although it is not clear how overexpression of C-FADD relates to the function of endogenous FADD, our study is consistent with recent data on mice carrying a FADD(S191D) replacement (the corresponding serine in mouse FADD), which show that regulation of FADD phosphorylation at Ser$_{194}$ is crucial for cell cycle progression in T cells (9). This situation is reminiscent of the remarkable similarities between T cells from C-FADD transgenic mice and FADD-deficient mice (5, 3). Collectively, the data point to an important role of the kinase that phosphorylates FADD on Ser$_{194}$ in linking death receptor signaling to cell cycle progression. In summary, our data demonstrate: first, the activity of C-FADD to affect cell cycle progression is not restricted to T cells; second, the serine 194 phosphorylation site of FADD is involved in this effect; and third, cells have different thresholds to respond to the cell cycle inhibiting effects of C-FADD.

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Fig. 3. Expression of a constitutive phosphorylation mimicking mutant C-FADD-SE increases efficiency of G$_2$/M arrest. A. BJAB control cells or BJAB cells stably transfected with C-FADD wild type (wt) or the point mutants S194A (SA) or S194E (SE) were treated with anti-CD95 (closed bars) or control treated (open bars). Apoptosis was quantified as the percentage of cells with DNA fragmentation. The inset is an anti-FADD Western blot showing the exogenous proteins. B. kinetics of G$_2$/M arrest in BJAB stable transfectants treated with 50 nM Taxol. C. anti-FADD Western blot corresponding to the 18-h time point in D, in normal cycling cells or cells arrested with Taxol. D. kinetics of G$_2$/M arrest in retrovirally transduced MCF10A-Myc cells stably expressing C-FADD wt, SA, or SE arrested with 500 nM Taxol. Kinetics of untransfected cells were similar to the wt- and SA-expressing cells (data not shown). The experiments are representative of at least four independent experiments.
Cell Cycle Effects by C-FADD Depend on Its C-terminal Phosphorylation Site
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