

Supplemental Data

Phosphorylation of FADD at Serine 194

by CKI α Regulates Its Nonapoptotic Activities

Elizabeth C. Alappat, Christine Feig, Jörg Volkland, Ben Boyerinas, Martin Samuels, Andrea E. Murmann, Andrew Thorburn, Vincent J. Kidd, Clive A. Slaughter, Stephanie L. Osborn, Astar Winoto, Wei-Jen Tang, and Marcus E. Peter

Supplemental Results

Testing FADD Kinase Candidates

We previously demonstrated that C-FADD was as efficiently phosphorylated at Ser194 as full-length FADD (Alappat et al., 2003; Scaffidi et al., 2000). Since both FADD and C-FADD were found to be phosphorylated in a cell cycle dependent manner, we first attempted to identify the FADD associated kinase by testing the activity of various kinases to phosphorylate a GST-C-FADD fusion protein. In addition to the previously tested cell cycle regulated kinases Cdk1/cyclin A, cdk2/cyclin A, cdk1/cyclin B1, and cdk2/cyclin E (Scaffidi et al., 2000) we also failed to phosphorylate C-FADD with the polo kinases Fnk, Plk-1 and Snk, RIP, Jnk1, Jnk2, p38, Cdk3, 4 and 6 in *in vitro* kinase assays (data not shown). It has been proposed previously that Hip kinase 3/FIST is involved in phosphorylation of FADD (Rochat-Steiner et al., 2000). We determined that neither FIST nor the other Hip kinases 1 and 2 can directly bind or phosphorylate FADD (data not shown).

Supplemental Experimental Procedures

Expression Constructs

The point mutant C-FADD(SA) contains an Ala mutation at Ser194 and C-FADD(mt) contains an Asn mutation at Val121. Both point mutants and deletion mutants $\Delta 2$ (aa 142-203) and $\Delta 3$ (aa 177-203) were generated using standard PCR and cloning techniques. The wt peptide used for substrate phosphorylation contained an antennapedia peptide (GRQIKIWFQNRRMKWKKN) fused to the C-terminal 20 residues of FADD (RSGAMSPMSWNSDASTSEAS). The SA peptide had an alanine residue replacing the underlined serine. GST-Axin refers to a GST-Axin (β Cat+S45K) fusion protein (Liu et al., 2002). CKI α LS was used as a GFP fusion protein in the pC1-neo vector (Fu et al., 2001). All AU1-FADD (full length) and AU1-C-FADD (aa 80-208)

constructs were in pcDNA3 and include a wild-type form (wt) described previously (Scaffidi et al., 2000) and point mutants SA (Ala mutation at Ser194) and AP (Pro mutation at Ala 174) generated by site directed mutagenesis (Quikchange Mutagenesis kit, Stratagene). All YFP fusion proteins were in the YFP-C1 vector and had an N-terminal YFP fused to C-FADD, and constructed as described previously (Morgan et al., 2001). To some of the YFP-C-FADD constructs the SV40 NLS was added (by fusing it to YFP).

Cell Transfections, Viral Transductions and Cell Extracts

Cells were transfected with RNAi oligonucleotides at a concentration of 40 nM using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were harvested 72 hours post transfection for Western blot analyses and kinase assays. For Taxol sensitivity assays, siRNA transfected cells were treated after 48 hours with Taxol and harvested at 18 hours for cell cycle analysis and at 14 hours for immunocytochemistry. Adenoviral transduction with AdV C-FADD(wt) was done as described before (Alappat et al., 2003). Whole cell lysates were made with 0.5% NP40 lysis buffer (10 mM Tris pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% glycerol, 1 mM PMSF and protease inhibitors (Roche)), followed by sonication and centrifuged at 14,000g. To produce nuclear extracts, cells were incubated for 20 min in a hypotonic buffer on ice (10 mM Hepes pH 7.4, 10 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol, and 1 mM PMSF). The cells were gently broken up using a Dounce homogenizer. Small aliquots of cells were stained with trypan blue to determine the progression of the lysis. Homogenization was continued until 95% of cells were broken. The broken cells were then centrifuged at 3000 g for 20 min. The pellet contained the nuclei and the supernatant, the cytosol. The pellet was then resuspended in lysis buffer, sonicated and centrifuged at 14,000 g. The supernatant fraction was the nuclear extract.

Protein Purification and Mass Spectrometry

Whole cell lysates were prepared from 1.7×10^{10} RIP^{-/-} Jurkat cells and applied to a 30 ml Q Sepharose High Performance column equilibrated in Buffer A (10 mM Tris pH 7.7, 1 mM EDTA, 20 mM NaCl, 1% glycerol and 1 mM PMSF). Bound proteins were eluted with a linear gradient of 0-1 M NaCl and FADD-K activity eluted at 300 mM NaCl. Active fractions (21-25) were pooled, concentrated to 1 ml and loaded onto a HiLoad 16/60 Superdex 75 column equilibrated in Buffer B (same as A, but 300 mM NaCl and 0.5% NP40). Active fractions (21-

23) from Superdex-75 were dialyzed against a basic buffer (30 mM citric acid pH 5.5, 20 mM NaCl, 1 mM EDTA, and 1% glycerol) and separated on a 17 ml SP Sepharose High Performance column. Proteins were eluted with a linear NaCl gradient and FADD-K activity eluted at 400 mM NaCl in fractions 36-38. All columns were from Amersham Pharmacia. Eluted fractions were subjected to SDS-PAGE electrophoresis and silver stain. The five peak fractions were each concentrated ten fold and proteins were separated by SDS-PAGE and again visualized by silver staining (data not shown). Only one protein at approximately 37 kDa peaked with the activity. This protein and other bands in this lane were identified by mass spectral analysis. Proteins were reduced and alkylated with iodoacetamide and a tryptic digest was prepared. The unfractionated digests were subjected to matrix-assisted laser desorption/ionization (MALDI) tandem time-of-flight (TOF/TOF) mass analysis on an Applied Biosystems Model 4700 Proteomics Analyzer. 10 peptides were assigned to CKI α .

Immunoprecipitation, Immunocytochemistry and Western Blot Analysis

For immunoprecipitations of AU1 tagged proteins or CKI α , cell lysates were incubated with protein A-Sepharose beads and 5-10 μ g of antibody for 2.5 hrs at 4°C. For the GST-C-FADD protein interaction assay 1 mg of GST or GST-C-FADD immobilized on glutathione beads was incubated with lysates from 2×10^8 Jurkat cells for 2.5 hours at 4°C to pull down associated proteins. The protein complexes were then stringently washed and subjected to immunoblot analysis. For immunocytochemistry, cells were grown on glass slide chambers (Fischer) and then fixed with a 1:1 ratio of methanol/acetone for 4 min at -20°C. Cells were then stained with the anti-phospho FADD antibody at 1:25 dilution for 16 hours at 4°C, followed by staining with secondary antibody and DAPI for 1 hr at room temperature. YFP expressing cells were fixed in 4% paraformaldehyde, permeabilized in TBS with 0.1% Triton X-100 and then stained with DAPI as described above. For laser scanning confocal microscopy HeLa cells were plated on glass slides and synchronized in medium without serum for 48 hours. After 18 hours of culture in complete medium containing 10% FCS cells were fixed with methanol/acetone and stained with antibodies against P-FADD, CKI α and α -tubulin (Serotec). All antibodies were used 1:25 in PBS for 1 h at room temperature. DNA was visualized by DAPI staining. Stained cells were analyzed using a Leica SP2 AOBS spectral laser scanning confocal microscope operated with the software LCS 2.5v1347.

***In vitro* Kinase Assay**

For FADD kinase assays, 3 μg of GST-C-FADD bound to glutathione Agarose beads (Pierce) was incubated with the source of the kinase: whole cell lysates, nuclear lysates, purified fractions, or purified CKI (Promega) for 1.5 hrs at 4°C. The bound substrate-kinase complex was washed three times with high salt lysis buffer (300 mM NaCl) and resuspended in 40 μl kinase buffer (65 mM Tris pH 7.5, 1% Triton, 10% glycerol, 100 mM NaCl, 10 mM MnCl_2 and 10 mM MgCl_2) with 10 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000Ci/mmmole, Amersham). After 30 min on ice the reaction was stopped with sample buffer and boiling. Alternately, when AU1-FADD or AU1-C-FADD were used as substrates, AU1 tagged proteins were immunoprecipitated along with bound kinase from transfected 293T cell lysates, stringently washed and incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in kinase buffer as described above. In cases where purified CKI was added, the immune complexes were further incubated for 1.5 hrs with the kinase, washed to remove unbound kinase and subjected to the phosphorylation reaction. For peptide phosphorylation reactions, 50 μg of peptide was added to the kinase reaction. CKI α activity assays were done as substrate phosphorylation reactions, where CKI α was immunoprecipitated from cell lysates and was resuspended in a final volume of 100 μl with kinase buffer 50 μg α -casein and 10 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 30 min on ice. For depletion experiments, nuclear lysate from 10^6 Jurkat cells in 200 μl was depleted sequentially with bead bound GST-C-FADD (300 μg /depletion) or GST-Axin (80 μg /depletion) or corresponding amounts of GST alone, for 1 hr at 4°C each. Progression of depletion was assayed by ability of bound kinase to phosphorylate GST-C-FADD itself or α -casein added to GST-Axin. Subsequent FADD kinase assays and CKI activity assays from depleted lysates were done as described above.

Supplemental References

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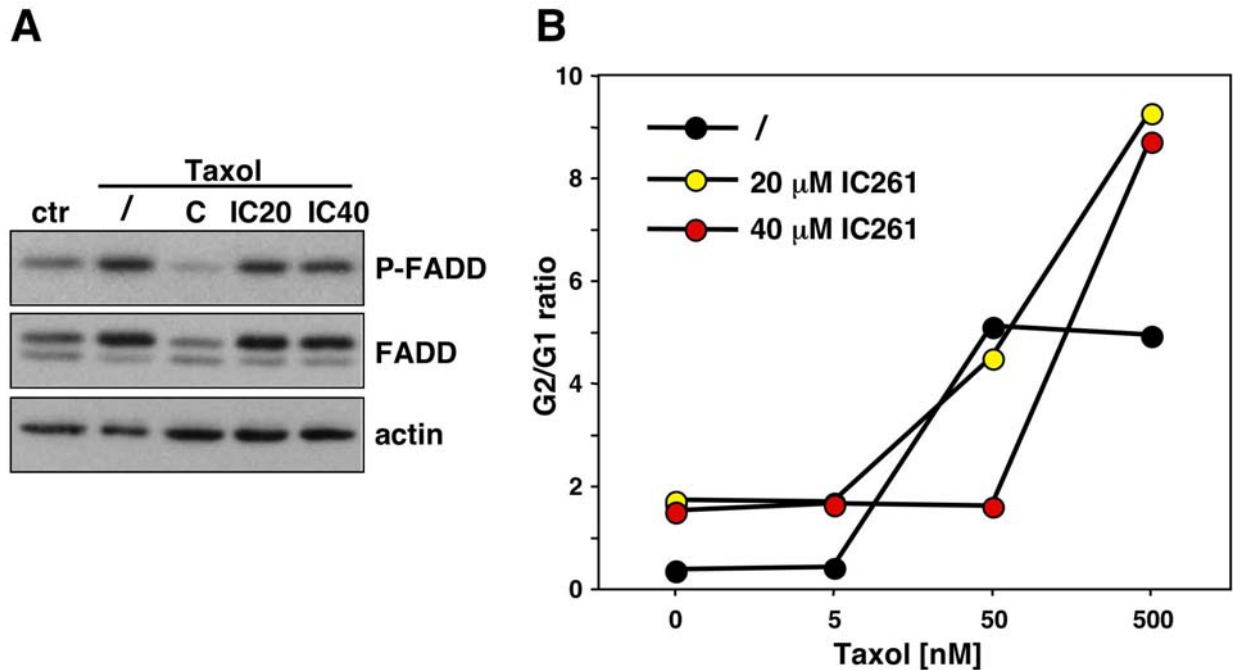


Figure S1. The CKI δ and ϵ Selective Inhibitor IC261 Does not Inhibit the Phosphorylation of FADD.

(A) Western blot analysis of HeLa cells for P-FADD, FADD and actin left untreated or treated with 50 nM of Taxol for 18 hrs in the presence of 250 μ M CKI-7 (C), 20 μ M IC20 or 40 μ M IC261 (IC40).

(B) Sensitivity to Taxol mediated G2/M arrest was measured in BJAB cells cultured with Taxol at the indicated concentrations for 18 hours in the presence of 0, 20 or 40 μ M IC261. Cell cycle analysis data is represented as the ratio of cells in G2 to G1.

indicating that ΔC contains the domain required for binding to FADD-K, and that a peptide that contains only the phosphorylation site is accepted by FADD-K as a substrate (lane 6). This phosphorylation was again specific for S194 since a peptide carrying the SA mutation was not phosphorylated in comparison (lane 9). In summary, the FADD-K binding domain on FADD comprises amino acids 142-189. K, *in vitro* kinase assay; C, Coomassie staining.

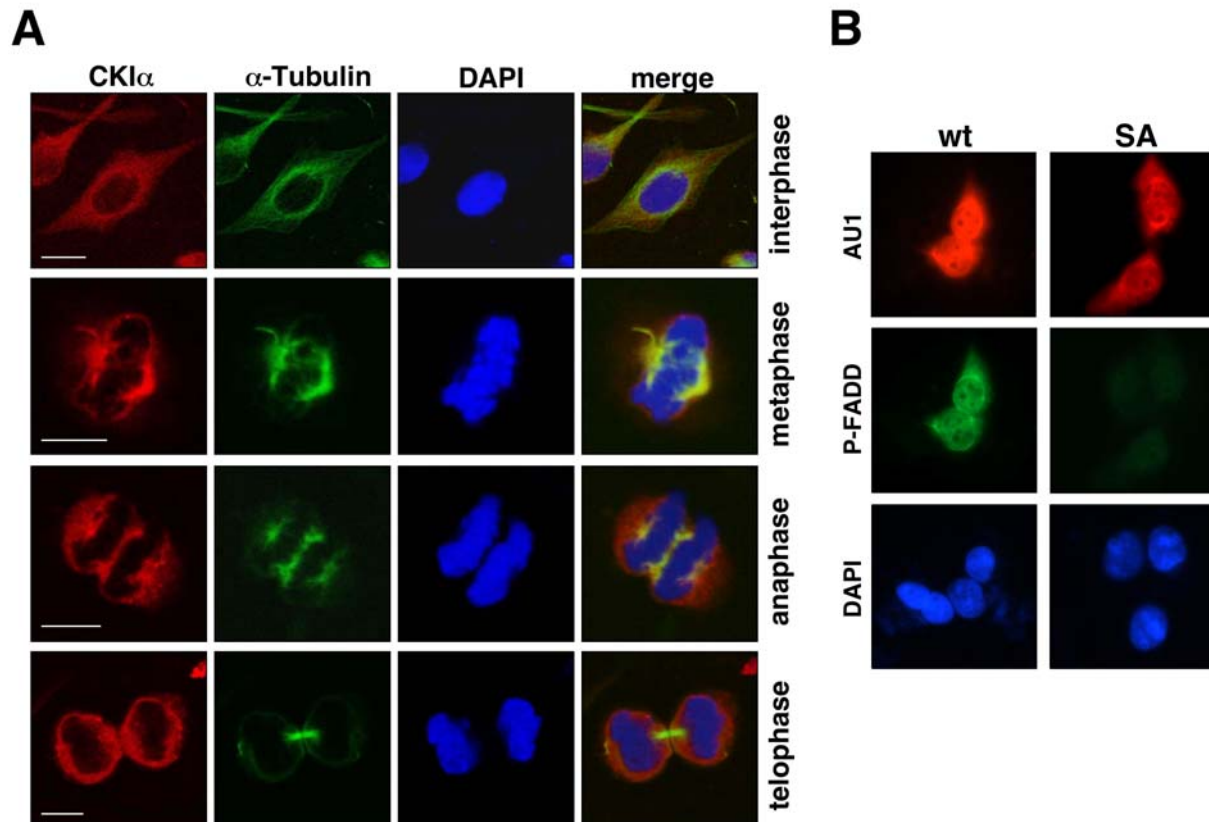


Figure S3. CKI α Colocalizes with α -tubulin on Mitotic Spindles during Meta and Anaphase.

(A) Confocal immunofluorescence analysis of HeLa cells in different stages of the cell cycle. Cells were stained for CKI α , α -tubulin and nuclei were visualized with DAPI as indicated. Bar, 10 μ M. Bar, 10 μ M.

(B) Establishing the specificity of the anti-phospho-FADD Ab to detect S194 phosphorylated FADD in immunofluorescence microscopy. 293T cells were transfected with either AU1-C-FADD (wt) or AU1-C-FADD(SA) and stained anti-AU1, anti-P-FADD or DAPI.