A Novel Juxtamembrane Domain in Tumor Necrosis Factor Receptor Superfamily Molecules Activates Rac1 and Controls Neurite Growth

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Members of the tumor necrosis factor receptor (TNFR) superfamily control cell fate determination, including cell death and differentiation. Fas (CD95) is the prototypical “death receptor” of the TNFR superfamily and signals apoptosis through well established pathways. In the adult nervous system, Fas induces apoptosis in the context of neuropathology such as stroke or amyotrophic lateral sclerosis. However, during nervous system development, Fas promotes neurite growth and branching. The molecular mechanisms underlying Fas-induced process formation and branching have remained unknown to date. Here, we define the molecular pathway linking Fas to process growth and branching in cell lines and in developing neurons. We describe a new cytoplasmic membrane proximal domain (MPD) that is essential for Fas-induced process growth and that is conserved in members of the TNFR superfamily. We show that the Fas MPD recruits ezrin, a molecule that links transmembrane proteins to the cytoskeleton, and activates the small GTPase Rac1. Deletion of the MPD, but not the death domain, abolished Rac1 activation and process growth. Furthermore, an ezrin-derived inhibitory peptide prevented Fas-induced neurite growth in primary neurons. Our results define a new domain, topologically and functionally distinct from the death domain, which regulates neuritogenesis via recruitment of ezrin and activation of Rac1.

INTRODUCTION

Members of the tumor necrosis factor receptor (TNFR) superfamily are homotrimeric cell surface receptors that mediate pleiotropic biological effects, including death, proliferation, and differentiation. These receptors have no intrinsic enzymatic activity, but they use adaptor proteins to activate a variety of signaling pathways, including the p42/44 and p38 mitogen-activated protein kinases (MAPKs), nuclear factor- κ B (NF- κ B), c-Jun NH₂-terminal kinase, and the canonical caspase cascade leading to apoptosis (Aggarwal, 2003). Fas, also known as CD95, is the archetypal death receptor of the TNFR superfamily (Nagata and Golstein, 1995). Its only endogenous ligand is Fas ligand (FasL; CD178). Fas was originally described as a lymphocyte receptor able to transduce apoptotic signals (Trauth *et al.*, 1989; Yonehara *et al.*, 1989). However, Fas signaling can promote proliferation, differentiation, cytokine secretion, and tissue

regeneration as well as cell death (Alderson *et al.*, 1993, 1994; Biancone *et al.*, 1997; Barcena *et al.*, 1999; Desbarats *et al.*, 1999; Tsutsui *et al.*, 1999; Desbarats and Newell, 2000; Shinohara *et al.*, 2000; Choi *et al.*, 2002; O'Brien *et al.*, 2002; Peter *et al.*, 2007). Fas is expressed principally in the immune system, where it regulates cell numbers by inducing apoptosis (Brunner *et al.*, 1995; Dhein *et al.*, 1995; Ju *et al.*, 1995). In the adult nervous system, neurons generally express very low or undetectable levels of Fas constitutively, but readily up-regulate Fas in response to injury, such as oxidative stress, trauma, ischemia, pharmacological toxicity, excitotoxicity, and during some neurodegenerative diseases (for review, see Lambert *et al.*, 2003). In the adult nervous system in the context of neuropathology, Fas can induce apoptosis, notably in models of stroke and amyotrophic lateral sclerosis (Martin-Villalba *et al.*, 2001; Raoul *et al.*, 2002). However, during embryonic development and in the early postnatal period, neurons coexpress Fas and FasL (French and Tschopp, 1996; Bechmann *et al.*, 1999; Zuliani *et al.*, 2006). In this context, activation of Fas by FasL does not induce apoptosis, but rather promotes branching in axons and dendrites (Sakic *et al.*, 1998; Zuliani *et al.*, 2006). Interestingly, Fas is expressed constitutively in human adult neural progenitor cells isolated from the subventricular zone and does not induce apoptosis in these neurons (Ricci-Vitiani *et al.*, 2004). Therefore, during development and in neural progenitor cells, Fas regulates neuronal morphology rather than apoptosis.

Molecular mechanisms of Fas-induced apoptosis are well defined (for review, see Peter and Krammer, 2003). The cytoplasmic adaptor protein Fas-associated death domain (FADD) associates with the death domain of Fas, followed by recruitment and activation of caspases-8 and/or -10. Together, Fas, FADD and caspase-8/10 form the death-induc-

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Abbreviations used: CRIB, Cdc42/Rac1-interactive binding; DD, death domain; ERK, p42/44 extracellular signal-regulated kinase; FADD, Fas-associated death domain; FasL, Fas ligand; ICD, intracellular domain; luc, luciferase; MPD, membrane proximal domain; NGFR, low-affinity p75 nerve growth factor receptor; PAK1B, p21-activated kinase 1 protein 1B; RBD, Rho binding domain; ROCK, Rho-associated kinase; TNFR, tumor necrosis factor receptor.

ing signaling complex that triggers apoptosis. In contrast, the upstream molecular events regulating Fas-mediated neurite growth are unknown. The p42/44 extracellular signal-regulated kinase (ERK) cascade, an MAPK pathway, has been implicated in Fas-mediated neuritogenesis in sensory neurons (Desbarats *et al.*, 2003), but it is not required for Fas-induced neurite branching in hippocampal neurons (Zuliani *et al.*, 2006). The domain(s) of Fas and the Fas-interacting molecule(s) controlling neurite growth and branching have not previously been identified.

Here, we show that Fas directly regulates morphology, in the absence of apoptosis, in cell lines and in primary cortical neurons from embryonic mice. We demonstrate for the first time that Fas activates the small GTPase Rac1. We define a novel membrane-proximal domain (MPD) of Fas that recruits ezrin; is essential for Fas-induced Rac1 activation and process growth; and is distinct from the death domain. This newly defined MPD is shared with other members of the TNFR superfamily, including the low-affinity p75 nerve growth factor receptor (NGFR) and TNFR2. Our findings elucidate the membrane-proximal molecular mediators of Fas-induced process growth in developing neurons, thereby defining a new nonapoptotic pathway through which members of the TNFR superfamily can regulate process growth and branching.

MATERIALS AND METHODS

Cell Lines and Transfections

We obtained SH-SY5Y and COS-7 cells from the American Type Culture Collection (Manassas, VA) and maintained them in DMEM supplemented with 10% fetal calf serum (FCS). We performed transient transfections in 80–90% confluent cultures in six-well tissue culture plates containing glass coverslips, by using 4.0 μ g of plasmid DNA and 10 μ l of Lipofectamine 2000 in 2.5 ml of DMEM/10% FCS. Transfection efficiency was 50–80% as determined by flow cytometry. For process quantification, we plated 6×10^4 cells/well onto poly-D-lysine (PDL)-coated 16-well chamber slides and transfected the cells with 0.2 μ g of plasmid and 0.5 μ l of Lipofectamine 2000 in Opti-MEM (Invitrogen, Carlsbad, CA). Media were changed after 4–6 h, and experiments were performed beginning 18 h after transfection.

Constructs

We amplified the full-length and death-domain deleted (Δ DD) human Fas sequences by polymerase chain reaction (PCR) amplification of Invitrogen cDNA clone ID 4514272 (accession no. BC012479) encoding human Fas isoform 1 (5' primer, GGAAGCGAATTCACCTCG; 3' primer for full length, GTTTTCGAATTCGACCAAGCTTGG; 3' primer for Δ DD, GGTGATATGAATTCGAAGTC). We cloned the amplified sequences into the EcoRI site of the pCMV-Tag4A vector (Stratagene, La Jolla, CA), to generate Fas-C'-terminal-FLAG-tagged proteins (Fas-FLAG and Δ DD-Fas-FLAG). We produced the Fas Δ 191–204 construct by PCR amplification of pCMV-Fas (5' primer, CAATTCCTAATTTGTTGGGTGGAAAACCAAGGTTCTCATGAATC; 3' primer, GATTCATGAGAACCCTTGGTTTCCACCCAAACAATTAGTGGAATTG). In all experiments, we used the pCMV-Tag4-luciferase control expression vector encoding luciferase-FLAG (Stratagene) as a transfection control.

Cell Death Assays

We plated 5×10^3 HEP1-6 or COS-7 cells or 10^4 Jurkat cells per well, in 96-well plates. HEP1-6 and COS-7 cells were transfected with 0.2 μ g of Fas-FLAG or luc-FLAG control plasmids in 0.5 μ l of Lipofectamine 2000. Twenty-four hours after transfection, we changed the media and added 50 ng/ml agonistic anti-human Fas antibody CH11 where indicated. Jurkat cells were not transfected and were treated with CH11 at the concentrations indicated. In Figure 8, Jurkat cells were pretreated for 30 min with SERLI or IETD (200 μ M) before stimulation with CH11. After an additional 24-h incubation, we determined cell viability by the colorimetric 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolol]-1,3-benzene disulfonate assay (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Jurkat cells were used as a positive control for CH11-mediated apoptosis in assays using HEP 1-6 or COS-7 cells, and in some experiments, agonistic anti-mouse Fas antibody (clone Jo2; 5 μ g/ml) was included as a positive control for maximal killing through endogenous Fas.

GTPase Activation Assays

We used the Cdc42/Rac1-interactive binding (CRIB) domain of human p21 activated kinase 1 protein 1B (PAK1B) as a specific probe for activated Rac1 and Cdc42 and the Rho binding domain (RBD) of Rho-associated kinase (ROCK) as a specific probe for activated RhoA, in glutathione transferase (GST) pull-down assays (Manser *et al.*, 1994). We cotransfected COS-7 cells with Fas-FLAG or control luciferase (luc)-FLAG, and with myc-tagged Rac1, Cdc42, or RhoA, kindly provided by Dr. N. Lamarche-Vane (McGill University, Montreal, Canada). For detection of endogenous Rac1 activation, we transfected cells only with Fas-FLAG or control luc-FLAG vectors. We lysed the cells in GTPase assay buffer (25 mM HEPES, pH 7.5, 1% NP-40, 10 mM MgCl₂, 100 mM NaCl, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride [PMSF], 5 mM NaF, 5 mM sodium orthovanadate, and 1 μ g/ml each aprotinin and leupeptin) 20 h after transfection, and incubated the cleared lysate with glutathione-Sepharose 4B beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) coupled to GST-CRIB or GST-RBD for 1 h at 4°C. We washed the beads four times, boiled them in 2 $\times$ SDS sample buffer, and then subjected the eluted proteins to electrophoresis on 12% polyacrylamide gels. We transferred the proteins to nitrocellulose membranes and blotted them with anti-myc antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) to detect transfected GTPases, or anti-Rac1 (Millipore, Billerica, MA) for endogenous Rac1. We quantified the ratio of activated GTPase (in the pull-down) to total GTPase (in the lysate) by densitometry, and calculated Fas-induced GTPase activity as follows: (activated/total GTPase in Fas-transfected cells)/(activated/total GTPase in luciferase-transfected cells).

Immunoblotting and Coimmunoprecipitation (coIP)

We lysed COS-7 cells grown to 90% confluence in 10-cm plates then transiently transfected with luc-FLAG or Fas-FLAG in 1.5 ml of modified radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 μ g/ml each aprotinin, leupeptin, pepstatin; 1 mM NaF; and 1 mM sodium orthovanadate). We centrifuged and precleared the lysates, and then we immunoprecipitated precleared lysates with 4 μ g/ml antibody (anti-FLAG M2 [Sigma-Aldrich], anti-ezrin clone 3C12 [Sigma-Aldrich], or mouse immunoglobulin G [IgG]1 isotype control [Santa Cruz Biotechnology]) for 2 h followed by a 1-h incubation with protein G beads at 4°C. We washed the beads four times, boiled them in 2 $\times$ SDS sample buffer, and then subjected the proteins to electrophoresis on 10% polyacrylamide gels. We transferred the proteins to nitrocellulose membranes and blotted them with anti-FLAG polyclonal antibodies (Sigma-Aldrich) or ezrin antibodies (clone 3C12; Sigma-Aldrich), followed by secondary horseradish peroxidase-conjugated antibodies (Bio-Rad, Hercules, CA), and developed the bands with ECL kit reagents (GE Healthcare).

GST-Fusion Proteins and Binding Assays

We designed a series of GST-Fas constructs composed of the transmembrane and cytoplasmic regions of Fas, either full length or with the deletions described above (Δ DD and Δ 191–204). We excised appropriate Fas fragments from the respective Fas-pCMV-Tag4A constructs using BamHI (restriction site 10 amino acids extracellular to the transmembrane domain) and EcoRI, ligated the fragments into pGEX4T-1 (Clontech, Mountain View, CA), and expressed the fusion proteins in DH5 α induced with 0.5 mM isopropyl- β -D-thiogalactopyranoside at 30°C for 1 h. We purified the GST-fusion proteins according to the protocol provided by GE Healthcare. We prepared lysates from COS-7 cells (4×10^6) in lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 1 mM dithiothreitol, 1 mM PMSF, 1 mM sodium orthovanadate, and Complete protease inhibitor tablets) and precleared the lysates with glutathione-Sepharose 4B beads (GE Healthcare). We incubated 10 μ g of GST-fusion protein bound to glutathione-Sepharose beads with equal volume of COS-7 cell lysate for 1 h at 4°C. Where indicated, we added the inhibitory peptide SERLI at 50–200 μ M. We washed the beads four times, boiled them in 2 $\times$ SDS sample buffer, and immunoblotted the resulting proteins for ezrin as described above. Immunoblots were stripped and re-probed for GST.

Primary E15 Cortical Neurons

We obtained embryonic day E15 embryos from timed pregnancies of C57BL/6 mice bred in our animal facility. We removed the frontal cortices, minced the tissue, and dissociated the cells by sequential trituration in 0.25% trypsin/0.2% DNase in S-MEM and then in Neurobasal medium/10% FCS. We plated the cells onto PDL-coated 16-well chamber slides (6×10^4 cells/well) or PDL-coated glass coverslips in 24-well plates (3×10^5 cells/well) in Neurobasal medium supplemented with N2 and B27 (Invitrogen), and we maintained the cultures in 5% CO₂ at 37°C. Where indicated, we transfected the cells with the full-length human Fas coding sequence or the luciferase control vector (0.8 μ g of DNA) with 0.5 μ l of Lipofectamine 2000 in Opti-MEM. Transfections were performed after 1 d in vitro.

Inhibitors and Antibody Treatments

Six hours after transfection, we changed the media and added agonistic anti-human Fas antibody CH11 (50 ng/ml; Millipore). For untransfected mouse cells, we added agonistic anti-mouse Fas antibody Jo2 (2 μ g/ml; BD Biosciences, San Jose, CA) to stimulate endogenous Fas. Inhibitors of mitogen-activated protein kinase kinase (MEK)/ERK (PD98059, 30 μ M; Calbiochem, San Diego, CA), of Fas/ezrin interaction (SERLI peptide, 200 μ M; GenScript, Piscataway, NJ), or dimethyl sulfoxide vehicle control, were added with the antibodies where indicated. Cells were cultured with the antibodies and/or inhibitors for a further 18 h before fixation. For SH-SY5Y cells analyzed in Figure 8, cells were transfected in six-well plates and then replated onto coverslips 16–20 h after transfection in media already containing SERLI peptide (200 μ M) and/or CH11 (50 ng/ml). Fresh inhibitor was added after 6 h, and cells were incubated for a total of 10 h with the inhibitor and/or CH11 before fixation.

Immunofluorescence and Confocal Microscopy

We fixed cells with 4% paraformaldehyde in phosphate-buffered saline (PBS) 18 h after transfection and then permeabilized the cells in 0.25% Triton X-100, blocked them in 10% normal goat serum, and incubated them with anti-FLAG polyclonal antibodies (Sigma-Aldrich) followed by anti-rabbit-Alexa 488 secondary antibodies (Invitrogen), anti-ezrin antibodies (3C12) followed by Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), and phalloidin conjugated with Texas Red or Alexa Fluor 647 (Invitrogen) to detect F-actin. We counterstained the nuclei with 4,6-diamidino-2-phenylindole and mounted the slides in Aqua-Poly/Mount (Polysciences, Warrington, PA). Images for analysis of process outgrowth were acquired on a fluorescence microscope (DM IRE2; Leica, Wetzlar, Germany) at 40 $\times$ magnification by using MetaMorph software (Molecular Devices, Sunnyvale, CA). Images for colocalization were obtained by laser-scanning microscopy (LSM3 PASCAL; Carl Zeiss, Jena, Germany) using LSM 3 PASCAL software version 3.2 for acquisition and image analysis.

Process Outgrowth and Morphometric Analysis

We quantified process growth in transfected cells using phalloidin labeling to define processes on cells staining positive for FLAG, to provide an unbiased

comparison between Fas-FLAG and control luc-FLAG-transfected cells. We traced and quantified all actin-positive processes on transfected cells by using MetaMorph software. We defined neurites or primary processes as any processes emerging from the cell body, and branches as any processes emerging from a neurite primary process. For multiple branches, the primary process was defined as the longest continuous path emerging from the cell body. We controlled for general neurotrophic or inhibitory effects of Fas, Fas agonists, and pharmacologic inhibitors by determining cell body area, and we found no significant differences for any treatment.

Statistical Analyses

We compared Fas-transfected and control-transfected or treated and untreated cells using two-tailed unpaired Student's *t* test for parametric data and Mann-Whitney tests for nonparametric data, and the effect of a series of treatments by one-way analysis of variance. Statistical analyses were performed using Instat GraphPad software (GraphPad Software, San Diego, CA). All figures show mean $\pm$ SE.

RESULTS

Fas Expression Induces Process Outgrowth

We transiently transfected FLAG-tagged full-length human Fas (Fas-FLAG) into a Fas-negative line of SH-SY5Y human neuroblastoma cells. We transfected control cells with luc-FLAG expressed in the same plasmid. We observed a spontaneous change in morphology in cells expressing Fas, but not in the control luciferase-expressing cells, consisting of increased number of processes per cell, and increased frequency of cells extending extremely long processes (Figure 1A). Untransfected and control-transfected SH-SY5Y cells typically produce fine, short spikes spontaneously, and Fas

Figure 1. Fas expression induced process growth in neuronal and nonneuronal cell lines. (A) SH-SY5Y cells transfected with control luciferase-FLAG (luc) or full-length human Fas-FLAG (Fas) were labeled with antibodies to FLAG (green) and with phalloidin to detect F-actin (red). Bar, 5 μ m. (B) Graphs show average $\pm$ SEM length of processes (left), number of processes per cell (middle), and process branching (right), in luc-transfected (white bars), Fas-transfected (pale blue bars), or Fas-transfected cells treated with the Fas agonist CH11 (dark blue bars). (C) Graphs show the effect of blocking ERK activation using PD98059 (PD). Cells were treated as described in B (white, pale blue, or dark blue solid bars), or they were additionally treated with PD98059 (stripped bars). (D) COS-7 cells were transfected and stained as described above. Bar, 5 μ m. (E) Fas transfection-induced process growth in COS-7 cells that was not altered significantly by Fas ligation with CH11 or by inhibiting the ERK pathway with PD98059.

Figure 2. Fas-induced process growth does not require the Fas death domain. (A) COS-7 cells transfected with Δ DD-Fas-FLAG extend processes ($23 \pm 0.8\%$ of COS-7 cells transfected with Δ DD-Fas show process growth, compared with $23 \pm 1.4\%$ transfected with full-length Fas-FLAG and $1.6 \pm 0.5\%$ with luc-FLAG). Cells were stained with antibodies to FLAG (green) and with phalloidin (red). Bar, $5 \mu\text{m}$. (B) COS-7 and mouse HEP1-6 cells were transfected with control luc-FLAG, full-length human Fas-FLAG, or Δ DD-Fas-FLAG, and they were treated with isotype control IgM antibody (white bars) or with CH11 agonistic anti-human Fas antibody (gray bars). COS-7 cells are resistant to Fas-induced apoptosis, whereas full-length Fas, but not Δ DD-Fas, kills apoptosis-sensitive hepatic HEP1-6 cells. Untransfected human Jurkat cells, which express large amounts of endogenous Fas, are included as a Fas-sensitive positive control. Results are expressed as percentage of viable cells compared with untreated cells.

expression significantly increased process density (from 1.5 ± 0.3 to 2.3 ± 0.2 processes/cell). Activation of the Fas receptor with agonistic antibodies to human Fas (clone CH11) triggered a further significant increase in the number of processes per cell in Fas-transfected cells, as well as a striking increase in process branching (Figure 1, B and C). Untransfected SH-SY5Y cells did not express detectable endogenous Fas (as determined by flow cytometry), and they did not respond to agonistic Fas antibody (data not shown).

We investigated whether Fas could also induce process extension in cells that do not normally grow processes. We transfected the monkey fibroblastoid line COS-7 with Fas-FLAG or luc-FLAG (Figure 1, D and E), and observed that $<2\%$ of untransfected or control-transfected cells demonstrated process extension, whereas $23 \pm 1.3\%$ of Fas-transfected cells showed process growth (Figure 1E). Addition of agonistic Fas antibody to the culture medium did not increase the percentage of cells with processes (Figure 1E). These results show that expression of Fas is sufficient to initiate process outgrowth.

We have previously found that the ERK pathway is involved in Fas-mediated neurite growth (Desbarats *et al.*, 2003). We inhibited ERK activation using the pharmacological MEK inhibitor PD98059. Inhibition of the ERK pathway did not change the frequency of COS-7 cells extending processes in response to Fas expression (Figure 1E). However, PD98059 prevented ligand-dependent morphological changes induced by Fas ligation in SH-SY5Y cells (Figure 1C). These data suggested that new processes arising from Fas expression alone, without receptor activation, did not require ERK activation. In contrast, ligand-dependent fine-tuning of process morphology required MEK/ERK function (Figure 1, C and E).

These results showed that Fas could initiate the growth of new processes through an ERK-independent pathway. We next sought to determine the mechanism underlying Fas-induced process growth.

Fas-induced Process Outgrowth Does Not Require the Fas Death Domain

The death domain of Fas is essential for recruitment of FADD and subsequent activation of the proapoptotic caspase cascade (Peter and Krammer, 2003; Zuliani *et al.*, 2006). The death domain consists of six antiparallel α -helices. Amino acids 234, 238, 240, and 244, located in helices 2 and 3, are contact residues required for FADD binding (Huang *et al.*, 1996). We produced a truncated FLAG-tagged Fas construct missing helices 2–6 of the death domain. This construct, Δ DD-Fas, consists of residues 1–229 of wild-type human Fas and is lacking all FADD binding residues. We found that Δ DD-Fas was able to induce process growth in COS-7 cells as efficiently as wild-type Fas: $23 \pm 1.4\%$ of cells transfected with Fas-FLAG grew processes, compared with $23 \pm 0.8\%$ of cells transfected with Δ DD-Fas-FLAG. These results demonstrate that the death domain is not required for Fas to initiate process growth (Figure 2A).

To confirm that the proapoptotic and process growth functions of Fas are independent, we transfected wild-type Fas-FLAG and Δ DD-Fas-FLAG into Fas apoptosis-sensitive HEP1-6 mouse hepatocyte cells. Mouse HEP1-6 cells transfected with human Fas-FLAG underwent significant cell death in response to agonistic anti-human Fas antibodies (clone CH11, as used to induce process growth described above), whereas hepatocytes transfected with Δ DD-Fas-FLAG remained viable (Figure 2B). COS-7 cells were not susceptible to Fas-induced death (Figure 2B). We have shown previously that Fas-positive SH-SY5Y cells are resistant to Fas-induced apoptosis, as are many types of primary neurons (Raoul *et al.*, 2002; Landau *et al.*, 2005). Our results show that Fas-FLAG is a functional, ligand-responsive proapoptotic molecule when expressed in cells sensitive to Fas-induced apoptosis, whereas Δ DD-Fas-FLAG has lost its proapoptotic function. In contrast, both constructs stimulated process growth in cells resistant to Fas-induced apoptosis. Interestingly, apoptosis induced by Fas-FLAG was ligand dependent (Figure 2B), whereas process growth initiated

by Fas expression did not require ligand (Figure 1), further suggesting that these two Fas functions are autonomous and mediated through different mechanisms.

Fas Expression Induces Rac1 Activation

Fas can initiate process growth without using its death domain and without activating ERK. In searching for an alternative molecular mechanism, we reasoned that process growth must involve cytoskeletal rearrangement (Meyer and Feldman, 2002), and it was therefore likely to involve members of the Rho GTPase family, which are essential for process extension and cell motility. These small GTPases consists of the RhoA, Rac1, and Cdc42 families, with RhoA principally responsible for stress fibers formation and cytokinesis, whereas Rac1 and Cdc42 tend to regulate neurite growth (Nikolic, 2002). We asked whether Fas expression in COS-7 cells would activate RhoA, Rac1, or Cdc42 (Figure 3). We used the CRIB domain of human PAK1B as a specific probe for activated Rac1 and Cdc42 in a GST pull-down assay (Manser *et al.*, 1994). This assay specifically detects the activated (GTP-bound forms) of Rac1 and Cdc42. In parallel, we used GST fused to the RBD of ROCK as a probe for activated RhoA. We cotransfected COS-7 cells with luciferase control or Fas, and with myc-tagged Cdc42, Rac1, or RhoA. We found that Fas expression specifically increased the activation of transfected Rac1, but it did not alter the amounts of activated Cdc42 and RhoA in the cell (Figure 3A). To determine whether Fas expression could also activate endogenous Rac1, we transfected COS-7 cells with lu-

ciferase control or Fas and then measured endogenous Rac1 activation using GST-CRIB assays. We found that Fas expression induced activation of endogenous Rac1 (Figure 3, B and C). These findings demonstrate a new functional interaction between Fas and Rac1 that may facilitate actin cytoskeletal remodeling.

Fas Colocalizes and Physically Interacts with Ezrin

We hypothesized that ezrin may serve as a molecular link between the Fas receptor and Rac1 activation. Ezrin is a cytoplasmic molecule that links membrane-bound molecules to the cytoskeleton, and it has been implicated in Fas-induced apoptosis (Parlato *et al.*, 2000; Lozupone *et al.*, 2004; Fais *et al.*, 2005) as well as in Rac1 and Rho signaling (Bretscher, 1999; Ivetic and Ridley, 2004). We examined whether ezrin interacted with Fas during Fas-induced process growth, in the absence of apoptosis. We found that Fas and ezrin colocalized at the membrane of COS-7 cells transfected with Fas-FLAG, notably in the processes of Fas-expressing cells (Figure 4A). In contrast, luciferase-transfected cells showed only diffuse cytoplasmic colocalization of FLAG and ezrin staining, with no colocalization at the membrane, and no process growth (Figure 4A).

We performed coIP experiments to determine whether Fas physically interacted with ezrin. We detected ezrin in FLAG immunoprecipitates from cells expressing Fas-FLAG, but not from those transfected with control luc-FLAG (Figure 4B). We also found that Fas-FLAG, but not luc-FLAG, immunoprecipitated with endogenous ezrin (Figure 4B). These findings demonstrate that ezrin associates with Fas *in vivo*, in cells that are undergoing morphological differentiation rather than apoptosis.

Figure 3. Fas expression induces Rac1 activation. (A) Luciferase or Fas was coexpressed with myc-tagged Rac1, Cdc42, or RhoA in COS-7 cells. GST-CRIB binds activated Rac1 and Cdc42, and GST-RBD binds activated RhoA. Lysate lanes demonstrate total Rac1, Cdc42, or RhoA. (B) COS-7 cells were transfected with luciferase or Fas, and activation of endogenous Rac1 was determined using GST-CRIB pull-down assays. Total endogenous Rac1 was present at undetectable levels in the lysates; therefore, actin was used as a loading control. (C) Densitometry was performed on the immunoblots shown in A and B, and Fas-induced GTPase activation was determined as follows: (activated GTPase/total GTPase) in Fas-transfected cells/(activated GTPase/total GTPase) in Luc-transfected cells. Actin was used as a surrogate for total endogenous Rac1. The graph is representative of five independent experiments showing Fas-induced Rac1 activation.

A Novel Domain of Fas Controls Interaction with ezrin and Is Required for Fas-induced Process Growth

We investigated whether the Fas-ezrin interaction was required for Fas-induced process formation. Ezrin tends to associate with juxtamembrane clusters of basic amino acids (Bretscher, 1999; Tsukita and Yonemura, 1999). The 14 amino acid stretch cytoplasmic to the transmembrane domain of Fas contains a large number of basic residues, KRK (191-193) and RKHRK (200-204), defining a putative ezrin binding region that we have called the Fas MPD. We produced a construct containing a deletion of this region (Δ 191-204Fas) (Figure 5A). To determine whether Fas-induced process growth correlated with Fas binding to ezrin, we produced GST fusion proteins of the intracellular domains of full-length Fas (ICD-Fas), ICD- Δ DDFas, and ICD- Δ 191-204Fas, and examined their ability to bind ezrin. We found that the intracellular domains of full-length Fas and of Δ DDFas associated with ezrin from COS-7 lysates in a GST pull-down assay (Figure 5B). However, the deletion of residues 191-204 abolished the interaction with ezrin (Figure 5B), indicating that Fas 191-204 is essential for association with ezrin. We transfected Δ 191-204Fas-FLAG into COS-7 cells in parallel with Fas-FLAG and Δ DDFas-FLAG, and found that Δ 191-204Fas-FLAG was unable to induce process growth (Figure 5, C and D), demonstrating that residues 191-204 of Fas are essential for Fas-induced process growth in these cells. As a control, we also produced a Fas construct lacking the entire intracellular domain (Δ ICD-Fas). This construct was poorly expressed and did not induce process growth (Figure 5C).

Previous reports have implicated the interaction between Fas and ezrin in apoptosis (Luciani *et al.*, 2004; Fais *et al.*, 2005; Haouzi *et al.*, 2005; Charrin and Alcover, 2006). We

Figure 4. Fas colocalizes and physically interacts with ezrin. (A) Confocal microscopy reveals colocalization of transfected Fas-FLAG (green), endogenous ezrin (red), and F-actin (blue) in COS-7 cells. Fas/ezrin/F-actin triple colocalization is white, and Fas/ezrin colocalization is yellow. Inset shows an enlarged view of the processes. Control cells transfected with luc-FLAG do not show colocalization of FLAG and ezrin. Bar, 5 μ m. (B) Lysates from luc-FLAG- or Fas-FLAG-transfected cells were immunoprecipitated (IP) with control IgG, anti-ezrin, or anti-FLAG antibodies, and the IP products were run on duplicate gels. The first gel was immunoblotted (IB) for ezrin (left) and shows that Fas-FLAG, but not luc-FLAG, coprecipitates ezrin. The second gel was immunoblotted with antibodies to FLAG (right), revealing that ezrin coprecipitates Fas-FLAG but not luc-FLAG. HC, heavy chain of IgG.

therefore determined whether deleting the ezrin-binding domain of Fas would result in a construct unable to induce apoptosis in Fas-sensitive cells. We found that Δ 191-204-Fas-FLAG did not induce significant apoptosis in HEP1-6 mouse hepatocyte cells, unlike wild-type Fas-FLAG, that mediated significant cell death in response to agonistic anti-human Fas antibodies (clone CH11) (Figure 5E). This result is consistent with previous findings implicating ezrin and Rac in Fas-mediated apoptosis (Luciani *et al.*, 2004; Fais *et al.*, 2005; Haouzi *et al.*, 2005; Charrin and Alcover, 2006; Ramaswamy *et al.*, 2007).

Our data define a new domain of Fas, the Fas MPD, and they show that the Fas MPD, but not the death domain, is required for Fas-induced process outgrowth.

The Fas MPD Is Required for Fas-induced Rac1 Activation

To examine the mechanistic link between recruitment of ezrin and Rac1 activation, we examined whether the Fas MPD was necessary for Fas-induced Rac1 activation. We transfected COS-7 cells with full-length Fas-FLAG, Δ DD-

Fas-FLAG, or Δ 191-204Fas-FLAG. We found that full-length Fas and Δ DD-Fas induced activation of cotransfected myc-tagged Rac1, but that Δ 191-204Fas, which lacks the MPD, did not activate Rac1 (Figure 6, A and B). These data show that Fas-induced Rac1 activation is independent of the death domain, and conversely, that the MPD is required for Fas-induced Rac1 activation.

Expression of Exogenous Fas Increases Neuritogenesis in Primary Neurons

We investigated whether primary neurons would also respond to Fas transfection with increased neurite growth. We transfected primary embryonic mouse cortical neurons with human Fas-FLAG or luc-FLAG, and we found that stimulation of transfected Fas with agonistic antibodies resulted in a higher frequency of neurons extending a greater number of neurites with significantly more branch points (Figure 7A). Stimulation of Fas did not result in longer individual neurites, but rather in neurons with more neurites and more branch points, resulting in significantly more neurite growth associated

Figure 5. Fas interacts with ezrin through a 14-amino acid membrane-proximal domain essential for Fas-mediated process growth. (A) The diagram shows the extracellular domain (ECD), transmembrane domain (TM), putative ezrin binding domain (MPD), and DD of full-length Fas, and the GST fusion proteins corresponding to the full-length intracellular domain of Fas (ICDFas), the death-domain deletion (Δ DDFas), and deletion of the MPD (Δ 191-204Fas). (B) GST pull-down of ezrin by using the intracellular domains of full-length Fas (ICDFas), Δ DDFas, and Δ 191-204Fas as bait show that ezrin interacts with full-length and Δ DD Fas, but not with Δ 191-204Fas. The blot was stripped and reprobed with anti-GST antibody. (C) Confocal microscopy shows that Δ DDFas, but not Δ 191-204Fas, induces process growth. Fas bearing a deletion of the entire intracellular domain (Δ ICDFas), included as a control, does not induce process growth. Colocalization of FLAG (green) and ezrin (red) is yellow; colocalization of ezrin and actin is pink. Inset shows close-up of processes (merge of Fas and ezrin channels without actin overlay). (D) Full-length and Δ DD Fas induce equivalent process growth in transfected COS-7 cells, but Δ 191-204 Fas fails to induce significant process growth compared with control luc-transfected COS-7 cells. (E) Full-length Fas, but not Δ 191-204Fas, induces significant death in Fas-sensitive HEP 1-6 mouse hepatocytes ($p = 0.03$; white bars, cells treated with IgM control antibody, gray bars, cells treated with CH11). The amount of cell death in response to CH11 in cells transfected with human Fas is comparable with the maximal cell death induced by the Jo2 anti-mouse Fas antibody (normalized to cells treated with control IgG), which stimulates endogenous Fas on the hepatocytes (black bar).

with each neuron (Figure 7B). Neurons transfected with control luc-FLAG did not grow more than five neurites or develop secondary branch points; however, neurons transfected with Fas-FLAG demonstrated up to 10 neurites, and they frequently displayed secondary branching.

Our data suggest that Fas expression does not affect neurite elongation, but it does stimulate the initiation of new neurite growth, both from the cell body and by inducing new branch points. These findings are consistent with previous reports (Desbarats *et al.*, 2003; Zuliani *et al.*, 2006)

Figure 6. The MPD is required for Fas-induced Rac1 activation. (A) Full-length Fas (wtFas), Δ DDFas, or Δ 191-204Fas was coexpressed with myc-tagged Rac1 in COS-7 cells. Activated Rac1 was pulled down with GST-CRIB and revealed by immunoblotting for myc. Immunoblots of lysates demonstrate total Rac1 expression and actin as a loading control. Results from two independent experiments are shown. (B) Densitometry was performed on the immunoblots shown in A, and Rac1 activation induced by each construct was determined by the ratio of total Rac1 to activated Rac1 for each of the two experiments shown.

suggesting that Fas stimulates neurite outgrowth and branching in primary neurons in vitro and in vivo.

Fas-induced Process Growth in Cell Lines and in Primary Cortical Neurons Is Prevented by Blocking Fas-Ezrin Interaction

To explore the physiological significance of the interaction of ezrin with endogenous Fas, we asked whether disrupting the ezrin-Fas interaction in SH-SY5Y cells and in mouse cortical neurons would alter neuritogenesis. We designed a peptide containing the ezrin sequence required for specific binding to Fas (Lozupone *et al.*, 2004) as an inhibitor of Fas-ezrin interactions. This peptide, SERLI, showed a dose-dependent inhibition of ezrin binding to Fas (Figure 8A). SERLI also significantly inhibited Fas-induced death in Jurkat T cells (Figure 8B), consistent with previous reports showing that the Fas-ezrin interaction is involved in Fas-mediated death (Luciani *et al.*, 2004; Fais *et al.*, 2005; Haouzi *et al.*, 2005; Charrin and Alcover, 2006). We found that treatment with the peptide inhibitor SERLI blocked Fas-induced process outgrowth and process branching in SH-SY5Y cells (Figure 8, C and D), suggesting that blocking the Fas-ezrin interaction prevents Fas-mediated process growth.

Stimulation of endogenous Fas with agonistic antibodies to mouse Fas (clone Jo2) resulted in increased neuritogenesis and neurite branching (Figure 8, E and F), as reported previously (Desbarats *et al.*, 2003; Zuliani *et al.*, 2006). We found that Fas-induced neurite branching in primary neurons was blocked by addition of the peptide inhibitor (Figure 8, E and F), showing that the interaction of endogenous Fas with ezrin is involved in Fas-induced neuritogenesis in primary neurons.

DISCUSSION

Here, we define the upstream molecular interactions underlying Fas-mediated process growth. We describe a new domain of Fas, consisting of 14 membrane-proximal amino acids, that is required for ezrin binding to Fas and subse-

Figure 7. Exogenous Fas expression and ligation induced neurite growth in primary neurons. (A) Primary cortical neurons from embryonic mice were transfected with luc-FLAG or human Fas-FLAG. Human-Fas-transfected cells were additionally treated with anti-human agonistic Fas antibody (CH11) where shown. CH11 does not cross-react with endogenous mouse Fas expressed on mouse neurons. Images show cortical neurons labeled with antibodies to FLAG (green) and with phalloidin to detect F-actin (red). (B) Neurite growth and branching was quantified for luc-transfected (white bars), Fas-transfected (gray bars), and Fas-transfected/CH11-stimulated neurons (black bars). Neurites were quantified after 2 d in vitro. Graphs show average $\pm$ SEM for neurite density expressed as number of neurites per transfected neuron (left), number of branches per transfected neuron (middle), and total summed length of all neurites per transfected neuron (right). p values were calculated using the Mann-Whitney test for nonparametric data.

density (E) and branching (F) were quantified after 18 h in culture. Stimulation of endogenous Fas induced significant neurite growth and branching, which were prevented by blocking Fas–ezrin interactions with SERLI. Graphs show average $\pm$ SEM of four replicate cultures.

quent Rac1 activation and process growth. We show that disrupting the association between endogenous Fas and ezrin in cell lines and in primary neurons from embryonic mice prevents Fas-mediated neurite growth. Our findings elucidate a novel Fas-initiated pathway consisting of the Fas MPD, recruitment of ezrin, and Rac1 activation, resulting in process growth. This novel pathway functions independently of the canonical apoptosis cascade consisting of the FADD and caspase activation, resulting in apoptosis.

Ezrin has previously been shown to associate with Fas during apoptosis, resulting in Fas polarization to membrane rafts and uropods, thus promoting efficient apoptosis in activated lymphocytes, CD4⁺ T cells during human immunodeficiency virus infection, and hepatocytes (Luciani *et al.*, 2004; Fais *et al.*, 2005; Haouzi *et al.*, 2005; Charrin and Alcover, 2006). Rac has also been implicated in the death of activated T cells (Ramaswamy *et al.*, 2007). Consistent with this, we found that deletion of the ezrin binding MPD of Fas prevents Fas-induced killing of Fas-sensitive hepatocytes and that blocking the interaction between Fas and ezrin with the SERLI peptide inhibits Fas-dependent apoptosis in Jurkat T cells. However, our findings also reveal that an interaction between Fas and ezrin does not necessarily result in apoptosis but that the outcome of Fas activation is likely context dependent, resulting in apoptosis in activated immune cells and the damaged adult brain, and in neuritogenesis in the developing brain. Ezrin is a member of the ezrin/moesin/radixin (ERM) family of proteins, well characterized

Figure 8. Disruption of the Fas–ezrin interaction prevents Fas-induced process outgrowth. (A) SERLI, a peptide designed to compete with ezrin for binding to Fas, inhibits ezrin binding to Fas in a GST pull-down assay. The graph shows the densitometric ratio of ezrin pulled down by the intracellular domain of Fas (ICDFas) to total ICDFas for peptide concentrations between 0 (no inhibition) and 200 μM (complete inhibition). (B) SERLI inhibits apoptosis in human Jurkat T cells stimulated with CH11. The graph shows decreasing viability of cells treated with CH11 alone (gray triangles), and significant protection from death with addition of SERLI (black circles) or IETD, a peptide inhibitor of caspase-8 (gray squares, dashed line). Averages and standard deviations are shown. (C and D) SH-SY5Y cells were transfected with full-length human Fas-FLAG (white bars) or with control luciferase-FLAG (black bars), cultured overnight, and then replated with CH11 and/or with SERLI, a competitive peptide inhibitor of Fas–ezrin interactions. Processes (C) and branch points (D) per cell were quantified after 10 additional hours in culture with stimulators and/or inhibitors. Graphs show average $\pm$ SEM of three replicate cultures. p values show that CH11 induced significant additional process growth and branching in Fas-transfected cells compared with luciferase-transfected cells and that SERLI significantly inhibited processes and branch points in Fas-transfected cells that were not treated with CH11, as well as in Fas-transfected cells treated with CH11. (E and F) Mouse embryonic cortical neurons were treated with antibody to endogenous mouse Fas (Jo2) and/or with SERLI. Neurite

molecules that cross-link the cortical actin cytoskeleton with membrane proteins and regulate downstream signaling through a number of mediators, including the Rho GTPases (Bretscher, 1999; Tsukita and Yonemura, 1999). The ERM proteins are essential for the formation of morphological structures such as microvilli, leading edge, and cleavage furrows. Furthermore, ezrin has been implicated in neurite branching and in neurite regeneration after injury, by linking the neural cell adhesion molecule L1 to F-actin in growth cones (Haas *et al.*, 2004; Cheng *et al.*, 2005). Fas and ezrin are both present in neurons in the developing brain, and persists postnatally in neuroproliferative areas (Gimeno *et al.*, 2004).

We have shown that the Fas MPD consists of a stretch of 14 membrane-proximal amino acids. This region, rich in basic amino acids, resembles the ezrin-binding domains of other ezrin-interacting molecules, including L1, β -dystroglycan, CD44, CD43, and intercellular adhesion molecule-2 (Bretscher, 1999; Tsukita and Yonemura, 1999; Cheng *et al.*, 2005). Ezrin has been reported to activate the small GTPases by freeing them from their inhibitor, Rho-GDP dissociation inhibitor (Takahashi *et al.*, 1997). Here, we showed that recruitment of ezrin by Fas allowed Rac1 activation and process growth, suggesting that Fas can remodel cell morphology through posttranscriptional mechanisms, independently of new gene transcription. We and others have reported previously that Fas ligation can activate the ERK pathway (Shinohara *et al.*, 2000; Ahn *et al.*, 2001; O'Brien *et al.*, 2002; Desbarats *et al.*, 2003; Tamm *et al.*, 2004), and

Fas-induced ERK activation has been implicated in neurite growth through induction of p35 expression and subsequent activation of the neuron-specific cyclin-dependent kinase 5 (cdk5) (Desbarats *et al.*, 2003). Interestingly, cdk5 phosphorylates ezrin, thereby promoting the association of ezrin with membrane proteins (Yang and Hinds, 2006). Here, we have shown that inhibition of MEK/ERK signaling does not prevent Fas-induced process formation, but rather it reduces the length and branching of processes. Together, these data suggest a model in which Fas binding to ezrin is sufficient to initiate process growth independently of new gene expression, whereas ligation of Fas by FasL can trigger ERK activation, leading to new gene expression that further promotes process elongation and branching via cdk5. Moreover, phosphorylation of ezrin by cdk5 may further promote Fas–ezrin interactions and Fas-mediated process growth.

The physiological function of Fas in the nervous system is being reevaluated in light of recent evidence demonstrating that it is far more than a death receptor (Desbarats *et al.*, 2003; Lambert *et al.*, 2003; Landau *et al.*, 2005; Zuliani *et al.*, 2006; Peter *et al.*, 2007). Fas was initially considered to be exclusively proapoptotic in the nervous system, by analogy with the immune system, where Fas was first described as a “death receptor” (Becher *et al.*, 1998). However, mice with little or no Fas expression (*lpr* mice) have normal numbers of neurons, despite massively increased numbers of lymphocytes (Watanabe-Fukunaga *et al.*, 1992), suggesting that Fas is not a regulator of physiological neuron death during development (Landau *et al.*, 2005; Zuliani *et al.*, 2006; Peter *et al.*, 2007). In contrast, axon and dendrite branching is reduced in hippocampal and cortical neurons in Fas-deficient *lpr* mice in vivo, consistent with a role for Fas in promoting neurite branching (Zuliani *et al.*, 2006). *Lpr-cg* mice, which express Fas that bears a point mutation that inactivates the death domain, also show impaired Fas-mediated neuronal branching (Zuliani *et al.*, 2006), suggesting that the mutation in *lpr-cg* Fas may affect its binding to ezrin. In vitro, Fas ligation by FasL or agonistic antibodies stimulates neurite growth (Desbarats *et al.*, 2003) and branching (Zuliani *et al.*, 2006) in primary neurons from embryonic and neonatal mice. FasL expression has been reported in neurons, and it may therefore induce autocrine neurogenesis in neurons in the developing brain. Our data provide insight into the initial molecular steps of Fas-mediated process formation, and they illustrate, for the first time, a new domain of Fas linking Fas to a nonapoptotic pathway.

Fas-induced process growth is likely to have consequences beyond nervous system development. Ezrin-mediated remodeling of cellular morphology has been implicated in tumor motility and metastasis (Curto and McClatchey, 2004; Fais, 2004); interestingly, Fas has also been implicated in tumor progression and invasiveness through activation of NF- κ B and other nonapoptotic pathways (Barnhart *et al.*, 2004; Peter *et al.*, 2005). Our findings show that Fas–ezrin interactions do not invariably result in cell death, and suggest that this pathway may provide a new mechanism for Fas-mediated cancer progression and metastasis in apoptosis-resistant tumor cells.

Finally, the MPD that we describe here for Fas is homologous with a similar sequence of juxtamembrane basic amino acids found in the *Drosophila* protein Wengen, the sole *Drosophila* TNFR superfamily homologue (Kauppila *et al.*, 2003). There is no death domain in Wengen, suggesting that MPD-mediated cytoskeletal remodeling may evolutionarily predate caspase-mediated apoptosis in TNFR superfamily members. Several other members of the TNFR superfamily, including p75 NGFR and TNFR2, also share regions

homologous with the Fas MPD. Interestingly, TNFR2 has been preferentially associated with proliferation, whereas TNFR1 has been predominantly implicated in cell death (Aggarwal, 2003).

In summary, we have described a new domain in Fas, shared by several other TNFR superfamily members, that regulates recruitment of ezrin, Rac1 activation, and process growth. The Fas MPD controls a newly defined molecular pathway of morphological differentiation in developing neurons.

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