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, differentiation, 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-κB (NF-κB), 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, Fas usually induces apoptosis, notably in models of stroke and amyotrophic lateral sclerosis (Martin-Villaalba 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-
Novel Domain of Fas Controls Process Growth

**GTPase Activation Assays**

We used the Cdc42/Rac1-interactive binding (CRIB) domain of human p21 activated kinase 1 (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 Cdc42, or Rac2, 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 μg/ml each aprotinin, leupeptin, and pepstatin; 1 mM NaF, and 1 mM sodium orthovanadate). We centrifuged and precleared the lysates, and then we immunoprecipitated precleared lysates with 4 μg/ml antibody (anti-FLAG M2 [Sigma-Aldrich], anti-ezrin clone 3C12 [Sigma-Aldrich], or mouse immunoglobulin G [IgG] 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× 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).

**Immunoblotting and Comunnunoprecipitation (coIP)**

We lysed COS-7 cells grown to 90% confluency in 10-cm plates then transfectedly 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 μg/ml each aprotinin, leupeptin, and pepstatin; 1 mM NaF, and 1 mM sodium orthovanadate). We centrifuged and precleared the lysates, and then we immunoprecipitated precleared lysates with 4 μg/ml antibody (anti-FLAG M2 [Sigma-Aldrich], anti-ezrin clone 3C12 [Sigma-Aldrich], or mouse immunoglobulin G [IgG] 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× 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).

**GSTM-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 (ΔΔD and ΔΔ419-204). We excised appropriate Fas fragments from the respective Fas-pCMV-Tag3α constructs using BamHI (restriction site 10 amino acids extracellular to the transmembrane domain) and EcoRI, ligated the fragments into pGEX-4T-1 (Clontech, Mountain View, CA), and expressed the fusion proteins in DH5α induced with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) at 30°C for 4 h. We purified GST fusion proteins according to the protocol provided by GE Healthcare. We prepared lysates from COS-7 cells (4 × 106) 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 1 μg 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 μM. We washed the beads four times, boiled them in 2× SDS sample buffer, and immunoblotted the resulting proteins for ezrin as described above. Immunoblots were stripped and reprobed 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 MEM and then in Neurobasal medium supplemented with N2 and B27 (Invitrogen), and we maintained these cultures in Neurobasal medium (10% FCS). We plated the cells onto PDL-coated 16-well chamber slides (6 × 103 cells/well) or PDL-coated glass coverslips in 24-well plates (3 × 105 cells/well) in Neurobasal medium supplemented with N2 and B27 (Invitrogen), and we maintained these cultures in Neurobasal medium (10% FCS). Where indicated, we transfected the cells with the full-length human Fas coding sequence or the luciferase control vector (0.8 μg of DNA) with 0.5 μl 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 μg/ml; BD Biosciences, San Jose, CA) to stimulate endogenous Fas. Inhibitors of mitogen-activated protein kinase kinase (MEK)/ERK (PD98059, 30 μM; Calbiochem, San Diego, CA), of Fas/ezrin interaction (SERLI peptide, 200 μM; 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 peptides for a further 18 h before fixation. 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 ± 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 luciferase-FLAG (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

![Graph](image-url)
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 transfected the monkey fibroblastoid line COS-7 with Fas-FLAG, or ΔDD-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 ΔDD-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.

**Figure 2.** Fas-induced process growth does not require the Fas death domain. (A) COS-7 cells transfected with ΔDD-Fas-FLAG extend processes (23 ± 0.8% of COS-7 cells transfected with ΔDD-Fas show process growth, compared with 23 ± 1.4% transfected with full-length Fas-FLAG and 1.6 ± 0.5% with luc-FLAG). Cells were stained with antibodies to FLAG (green) and with phalloidin (red). Bar, 5 μm. (B) COS-7 and mouse HEP1-6 cells were transfected with control luc-FLAG, full-length human Fas-FLAG, or ΔDD-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 ΔDD-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.

**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, ΔDD-Fas, consists of residues 1-229 of wild-type human Fas and is lacking all FADD binding residues. We found that ΔDD-Fas was able to induce process growth in COS-7 cells as efficiently as wild-type Fas: 23 ± 1.4% of cells transfected with Fas-FLAG grew processes, compared with 23 ± 0.8% of cells transfected with ΔDD-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 ΔDD-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 ΔDD-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 ΔDD-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 consist 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 luciferase 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 co-IP 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.

### 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 (Δ191-204Fas) (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-Fas lacking the entire intracellular domain (ICDΔDD-Fas), and ICD-Δ191-204Fas, and examined their ability to bind ezrin. We found that the intracellular domains of full-length Fas and of ΔDD-Fas 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 Δ191-204Fas-FLAG into COS-7 cells in parallel with Fas-FLAG and ΔDD-Fas-FLAG, and found that Δ191-204Fas-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 (AICD-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...
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 Δ191-204-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, ΔDD-Fas-FLAG, or Δ191-204Fas-FLAG. We found that full-length Fas and ΔDD-Fas induced activation of cotransfected myc-tagged Rac1, but that Δ191-204Fas, 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 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 μm. (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.
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).
suggesting that Fas stimulates neurite outgrowth and branching in primary neurons in vitro and in vivo.

Figure 6. The MPD is required for Fas-induced Rac1 activation. (A) Full-length Fas (wtFas), ΔDDFas, or Δ191-204Fas 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.

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 ± 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.

**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-
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 ± 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 (J02) and/or with SERLI. Neurite 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 ± 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 rafis 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 neurogenesis in the developing brain. Ezrin is a member of the ezrin/moesin/radixin (ERM) family of proteins, well characterized 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. The peptide, which 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-mediated cancer progression and metastasis in apoptotic cells that this pathway may provide a new mechanism for interactions do not invariably result in cell death, and suggest that this sequence of juxtamembrane basic family, 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 novel 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.

ACKNOWLEDGMENTS

We thank R. Siegriest-Johnstone and C. Young for technical assistance; N. Lamarche-Vane for reagents, protocols, and advice on the GTPase activation assays; and E. Cooper, A. Fournier, and J. White for critical comments on the manuscript. This work was supported by Canadian Institutes of Health Research (CIHR) grant 53337 (to J.D.) and a New Investigator operating grant from Parkinson Society Canada (to J.D.) W.R. was supported by postdoctoral fellowships from CIHR and the Parkinson Society Canada, and J.D. was supported by a New Investigator Salary Award from CIHR.

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