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Receptor-interacting Protein Shuttles between Cell Death and Survival Signaling Pathways

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Cross-talk between apoptosis and survival signaling pathways is crucial for regulating tissue processes and mitigating disease. We report that anoikis—apoptosis triggered by loss of extracellular matrix contacts—activates a CD95/Fas-mediated signaling pathway regulated by receptor-interacting protein (RIP), a kinase that shuttles between CD95/Fas-mediated cell death and integrin/focal adhesion kinase (FAK)-mediated survival pathways. RIP's death domain was critical for RIP and Fas association to mediate anoikis. Fas or RIP attenuation reduced this association and suppressed anoikis, whereas their overexpression had the reverse effect. Overexpressing FAK restored RIP and FAK association and inhibited anoikis. Thus, RIP shuttles between CD95/Fas death and FAK survival signaling to mediate anoikis.

INTRODUCTION

Although communication between cell death and survival signaling pathways is critical for the regulation of many cellular and tissue processes, the molecules that mediate this cross-talk have not been well explored. Nuclear factor (NF)-κB induces the expression of antiapoptotic proteins and inhibits cell death pathways (Wang *et al*., 1996, 1998); however, other specific molecules that directly shuttle between cell death and survival processes have not been identified. We sought to explore this realm by examining the communication between the cell death receptors and integrin-mediated prosurvival signals. Death receptors such as tumor necrosis factor (TNF) receptor-1, DR3, DR4, DR5, and Fas/CD95 contain a cytoplasmic death domain and trigger apoptosis upon binding to their cognate ligands or specific agonistic antibodies. Activation of death receptors, such as Fas/CD95 leads to the formation of a death-inducing signaling complex (Krammer, 1999, 2000). Cancer cells, including squamous cell carcinoma (SCC) cells, constitutively express death receptors, such as Fas, and undergo apoptosis in response to treatment with Fas ligand (FasL) or Fas agonistic antibody (Moers *et al*., 1999; Shibakita *et al*., 1999). Signal transduction pathways for death receptors are well studied, and many signaling molecules are known to mediate death receptor-induced apoptosis (Peter and Krammer, 1998). However, the role of death receptors in extracellular matrix (ECM)-regulated anoikis, a form of apoptosis induced by loss of cell–ECM contacts, has not been explored.

Focal adhesion kinase (FAK) is important in signal transduction pathways that regulate cell survival and thereby tumorigenesis. Its expression is enhanced in laryngeal SCC (Aronsohn *et al*., 2003) and invasive colon and breast tumors (Owens *et al*., 1995). Different FAK domains are involved in its binding to cell surface integrin receptors and in recruiting to focal adhesions other proteins that mediate survival signals. Phosphorylation of FAK mediates downstream signaling events that regulate survival (Schlaepfer *et al*., 1994). Also, overexpression of constitutively active FAK rescues epithelial cell lines from anoikis (Frisch *et al*., 1996), whereas inhibition of FAK by antisense techniques triggers apoptosis (Xu *et al*., 1996). Numerous studies have shown a protective role for FAK in apoptosis (Schaller, 2001). In addition, FasL and Fas are constitutively expressed in SCC, and SCC cells undergo apoptosis upon treatment with a FAS agonistic antibody (Moers *et al*., 1999; Shibakita *et al*., 1999). Moreover, detachment of SCC cells from the ECM leads to suppression of FAK-mediated survival signaling, resulting in anoikis (Zhang *et al*., 2004). We hypothesize that anoikis in these cells is regulated by cross-talk between FAK survival pathways and death receptor-mediated cell death pathways. However, the mediators and the mechanisms of this cross-talk are not known.

Receptor-interacting protein (RIP), a kinase that encodes a unique domain with homology to serine/threonine and tyrosine kinases, is constitutively expressed in many tissues (Stanger *et al*., 1995). RIP interacts with Fas/CD95 in yeast and cancer cell death pathways (Stanger *et al*., 1995). Through its interactions with the death receptors TNF receptor 1, TNF receptor-associated factor 2, and TNF receptor type 1-associated death domain protein, RIP plays a pivotal role in regulating NF-κB (Meylan and Tschopp, 2005; Jackson-Bernitsas *et al*., 2007). RIP also interacts with the epidermal growth factor receptor and thereby contributes to activation of NF-κB by epidermal growth factor (Habib *et al*., 2001). In cell models, RIP is critical in NF-κB activation induced by the death receptor TNF-related apoptosis-inducing ligand (TRAIL) and by DNA-damaging

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agents (Lin *et al.*, 2000; Meylan and Tschopp, 2005). RIP also contributes to TNF-induced activation of c-Jun NH_2-terminal kinase and extracellular signal-related kinase (ERK) (Devin *et al.*, 2003). Furthermore, RIP interacts with FAK to suppress apoptosis and contributes to TNF-α–mediated activation of NF-κB (Kurenova *et al.*, 2004). In vivo, RIP plays a role in apoptosis as supported by the findings in RIP null mice (Kelliher *et al.*, 1998). Thus, RIP seems to participate in both cell survival and cell death pathways (Meylan and Tschopp, 2005); however, its ability to interplay between cell death receptor pathways and survival pathways has not been explored. Previously, we showed that an altered fibronectin matrix (Kapila *et al.*, 1997) induces anoikis of human SCC cells by suppressing integrin α v-mediated phosphorylation of FAK and ERK (Kamarajan and Kapila, 2007). In this study, we examined the link between the death receptor and survival integrin/FAK signaling pathways in regulating anoikis. Identification of key signaling mediators in this process could be useful in identifying therapeutic targets for the treatment of SCC.

MATERIALS AND METHODS

Cell Lines and Culture

The highly invasive human oral SCC cell line HSC-3 (Owens *et al.*, 1995) was kindly provided by Randy Kramer (University of California, San Francisco, San Francisco, CA). The human SCC cell line UM-SCC1 was a gift from Tom Carey (University of Michigan, Ann Arbor, MI). $RIP^{+/+}$ and $RIP^{-/-}$ mouse embryonic fibroblasts (MEFs) were kindly provided by Philip Leder and Michelle Kelliher (Harvard Medical School, Boston, MA). Wild-type and $FAK^{-/-}$ MEFs were from American Type Culture Collection (Manassas, VA). Fibroblasts from wild-type (Fas) and homozygous lymphoproliferation spontaneous mutation (Fas^{lpr}) mice (000482; The Jackson Laboratory, Bar Harbor, ME) were isolated from skin by standard methods. Cells were maintained in a 5% CO_2 atmosphere at 37°C in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin.

Plasmids and DNA Constructs

The FAK construct (pRc/CMV-FAKHA) was provided by Steven K. Hanks (Vanderbilt University, Nashville, TN). FLAG- or myc-tagged wild-type RIP and myc-tagged RIP deletion mutants, including $RIP_{1\text{-}558}$ and $RIP_{559\text{-}671}$, have been described previously (Inohara *et al.*, 2000; Lin *et al.*, 2006). The FasGFP (pEF-GFPFas) construct was provided by Ze'ev A. Ronai (Burnham Institute, La Jolla, CA).

Inducing Anoikis with an Altered Fibronectin Matrix

Cells were treated with a recombinant fibronectin (FN) protein (Kapila *et al.*, 1999) containing an alternatively spliced V region and a mutant, nonfunctional [V^+H^-] high-affinity heparin-binding domain (Barkalow and Schwarzbauer, 1991). This protein also contains the RGD cell binding site and the alternatively spliced EIIIA domain. The mutant V^+H^- protein (mtFN) alters the fibronectin matrix, rendering it dysfunctional and inducing anoikis (Kapila *et al.*, 1999; Tafolla *et al.*, 2005; Kamarajan and Kapila, 2007; Joo *et al.*, 2008). Thus, anoikis conditions were set by treatment with V^+H^- or mtFN.

Flow Cytometry

The percentage of apoptotic cells was determined by flow cytometry. In brief, cells were grown in 12-well plates and treated as indicated. Adherent cells were detached by incubation with enzyme-free dissociation buffer (Invitrogen), pelleted by centrifugation, and stained with annexin V-fluorescein isothiocyanate (FITC) or annexin V-phycoerythrin (BD Biosciences Pharmingen, San Diego, CA) for analysis by flow cytometry (FACSDiVA Cell Sorter; BD Biosciences, Franklin Lakes, NJ). For surface expression, cells were washed twice with phosphate-buffered saline (PBS) and stained with monoclonal antibodies (mAbs) to Fas (mAb 3061), FasL (mAb 3912; Millipore Bioscience Research Reagents, Temecula, CA and sc-71097; Santa Cruz Biotechnology, Santa Cruz, CA), TNFR1 (sc-73195; Santa Cruz Biotechnology), or DR5 (sc-53688; Santa Cruz Biotechnology), followed by secondary antibodies conjugated with FITC, and then analyzed and quantified by FACScan (BD Biosciences). Fold changes were assessed relative to control. DNA fragmentation was measured using the cell death assay kit (11774425001; Roche Molecular Systems, Alameda, CA).

Immunoprecipitation and Immunoblot Analysis

For immunoprecipitation experiments, cells were treated as indicated, and lysed in radioimmunoprecipitation assay lysis buffer containing protease inhibitors (50 mM Tris/HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1% protease inhibitor cocktail [P8340; Sigma-Aldrich, St. Louis, MO), 1 mM Na_3VO_4, and 1 mM NaF), and immunoprecipitation was carried out by standard methods. Western blotting was performed with various antibodies. Antibodies for Fas (sc-8009 and sc-715), c-myc (sc-40), green fluorescent protein (GFP; sc-8334, sc-9996), and β-actin (sc-1615) were from Santa Cruz Biotechnology. Antibodies for FAK (05-182), caspase-8 (9746), FLAG (M2-A8592), and RIP (610459) were from Millipore (Billerica, MA), Cell Signaling Technology (Danvers, MA), Sigma-Aldrich, and BD Biosciences Pharmingen, respectively. Fas-associated death domain (FADD) antibody was from Millipore. All other reagents were from Sigma-Aldrich.

Transient Transfection of Cells and Establishment of Stable Cell Lines

Cells at 60–70% confluence in twelve- or six-well plates were transiently transfected with small interfering RNA (siRNA) for Fas or RIP or with cDNA for Fas (1.0 μg), FAK (1.0 μg), FLAG-tagged RIP (1.0 μg), myc-tagged wild-type RIP, RIP deletion mutants (1.5 μg), or control vectors in 0.5 or 1 ml of serum-free medium containing Lipofectamine Plus (Invitrogen) by standard methods. Transfection efficiency was assessed by measuring the levels of Fas, FAK, FLAG, RIP, or c-myc in transfected and control cells by Western blotting or GFP fluorescence, and it ranged between 60 and 70%. For the production of stably transfected cell lines, Fas^{lpr} cells were transfected with pEF-GFP (Addgene plasmid, 11154) or pEF-GFP-Fas in 0.5 ml of serum-free media containing Lipofectamine Plus by standard methods. The cells were split 1:20 into fresh medium at 24 h after transfection. Transfected cells were cultured in selective medium containing 300 μg/ml geneticin antibiotic (10131-035; Invitrogen) for an additional 10 d. The surviving cell colonies were picked and propagated in a fresh 12-well dish before testing for transgene expression.

Statistical Analysis

In general, values are expressed as means ± SD. Intergroup differences were determined by two-way analysis of variance and Scheffé's multiple-comparison test. Statistical significance was defined as *, #$p \leq 0.01$. All experiments were repeated at least three times.

RESULTS

Anoikis Mediated by Fas/CD95 Signaling, Triggers Association of RIP with Fas and Dissociation of RIP from FAK

To determine whether death domain containing receptors are involved in anoikis of SCC cells, we examined the levels of the major classes of death domain-containing receptors Fas, TNFR1, and DR5 under anoikis conditions. Anoikis conditions were induced by treatment with altered fibronectin (mtFN) or incubation under suspension culture conditions, and then cell surface expression of the death domain containing receptors was specifically assessed by flow cytometry. Fas and its cognate ligand, FasL, but not TNFR1 levels were increased significantly (Figure 1A). Although DR5 expression was increased under anoikis conditions, the degree of induction was less than that of Fas. Suppression of Fas with neutralizing antibodies or silencing Fas with siRNA rescued SCC cells from anoikis, as assessed morphologically and by flow cytometry (Figure 1A, right; and B, left and middle). In addition, Fas suppression inhibited DNA fragmentation in SCC cells (Figure 1B, right). Suppression of Fas by siRNA was confirmed by Western blot analysis. Furthermore, death-inducing signaling complexes were formed under anoikis conditions, as evidenced by the increased association between Fas and FADD and between Fas and caspase-8, indicating Fas activation (Figure 1C).

To determine how anoikis modulates survival signaling in the context of Fas activation, we investigated the role of FAK. Because RIP interacts with FAK and Fas independently to regulate survival and death signaling pathways, we examined those interactions in SCC cells under anoikis

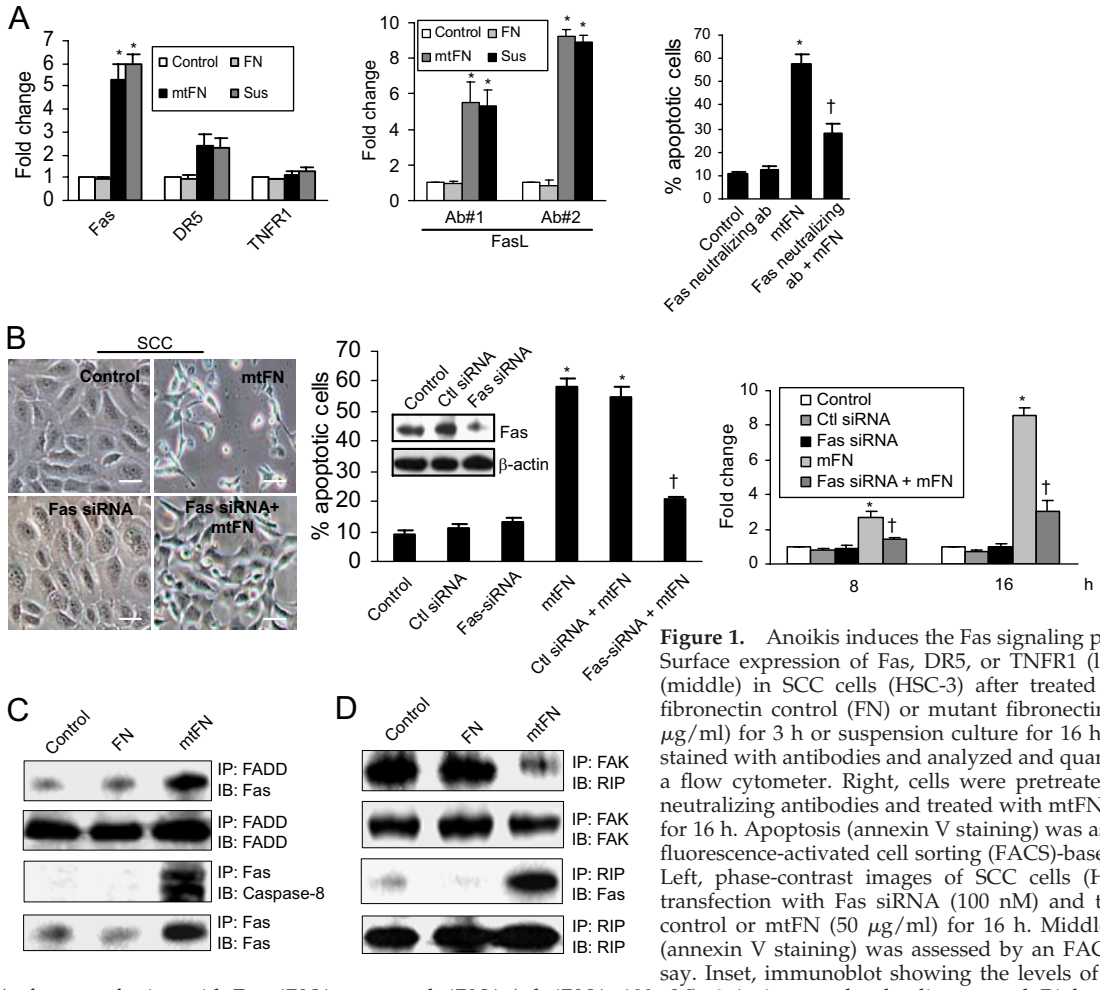

Figure 1. Anoikis induces the Fas signaling pathway. (A) Surface expression of Fas, DR5, or TNFR1 (left) or FasL (middle) in SCC cells (HSC-3) after treated with intact fibronectin control (FN) or mutant fibronectin (mtFN; 50 μg/ml) for 3 h or suspension culture for 16 h. Cells were stained with antibodies and analyzed and quantified using a flow cytometer. Right, cells were pretreated with Fas-neutralizing antibodies and treated with mtFN (50 μg/ml) for 16 h. Apoptosis (annexin V staining) was assessed by a fluorescence-activated cell sorting (FACS)-based assay. (B) Left, phase-contrast images of SCC cells (HSC-3) after transfection with Fas siRNA (100 nM) and treated with control or mtFN (50 μg/ml) for 16 h. Middle, apoptosis (annexin V staining) was assessed by an FACS-based assay. Inset, immunoblot showing the levels of Fas in SCC cells (HSC-3) after transfection with Fas siRNA or control siRNA (ctl siRNA; 100 nM). β-Actin served as loading control. Right, fold change of DNA fragmentation in SCC cells (HSC-3) after transfection with Fas siRNA (100 nM) and treated with control or mtFN (50 μg/ml) for 8 or 16 h. (C and D) SCC cells (HSC-3) were treated with control or mtFN (50 μg/ml) for 3 h, and lysates were immunoprecipitated and immunoblotted as indicated. Immunoglobulin G (IgG), in the cases when it was used, it did not overlap with the proteins of interest. Bars, 50 μm. *, #p $\leq$ 0.01.

conditions. Interactions with FAK decreased, whereas those with Fas increased (Figure 1D), suggesting that RIP shuttles back and forth between FAK and Fas to regulate anoikis.

Fas Signaling and Association with RIP Is Required for Anoikis

To determine the role of Fas in regulating anoikis and to explore interactions between cell survival and cell death signaling pathways, we examined Fas^{lpr} fibroblasts under anoikis conditions. The cells were resistant to anoikis as assessed morphologically and by flow cytometry (Figure 2A), showing Fas mediates anoikis. In contrast, Fas^{lpr} fibroblasts overexpressing Fas became sensitized to apoptosis under anoikis conditions (Figure 2B), confirming that Fas is an important mediator in matrix-regulated anoikis. Coimmunoprecipitation data confirmed that RIP dissociated from FAK and associated with Fas to mediate anoikis (Figure 2C). Overexpression of GFP-Fas was confirmed by Western blot analysis, and its membrane localization was also confirmed in GFP-Fas–transfected cells (Figure 2, B and C). Furthermore, Fas^{lpr} cells were stably transfected with empty vector or Fas and resultant clones were designated as GFP-Fas^{lpr} and GFP-Fas-Fas^{lpr}, respectively. Fas overexpression was confirmed at the protein level using Western blotting against the GFP tag (Figure 2D, inset). Fas overexpression resulted in a significant increase in DNA fragmentation under anoikis conditions, whereas, cells expressing empty vector did not (Figure 2D).

RIP Shuttles between FAK and Fas to Regulate Anoikis

We hypothesized that RIP shuttles between a FAK-bound state to promote survival signaling and a Fas-bound state to promote death signaling pathways. To examine this possibility, we tested FAK-null (FAK$^{-/-}$) MEFs under anoikis conditions. The FAK$^{-/-}$ cells were highly sensitive to anoikis. Overexpression of FAK in transfected FAK$^{-/-}$ cells inhibited anoikis (Figure 3A). FAK expression in the transfected cells was confirmed by Western blot analysis (Figure 3A, inset). Next, we examined the association of RIP with FAK and Fas. Consistent with our hypothesis, RIP strongly associated with FAK upon FAK overexpression, and this association was diminished by treatment with mtFN under anoikis conditions (Figure 3B). Conversely, interactions between Fas and RIP were not apparent upon FAK overexpression, but they increased in response to treatment with mtFN (Figure 3B).

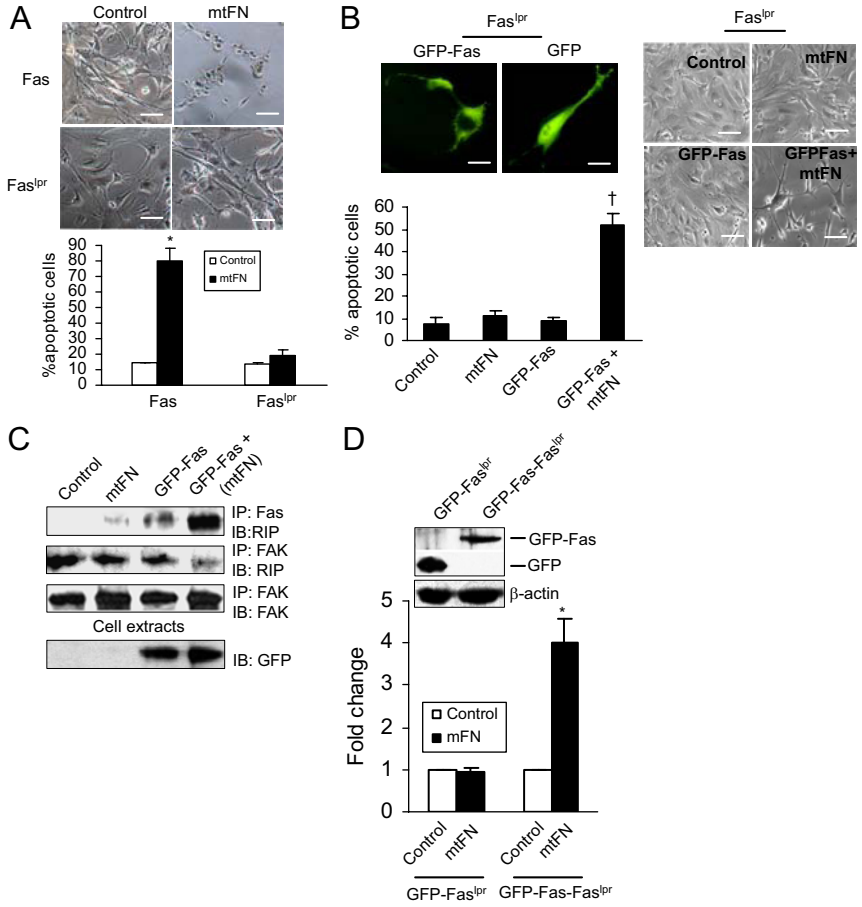

Figure 2. Fas signaling and association with RIP is required for anoikis. (A) Top, phase-contrast images of wild-type (Fas) and Fas-deficient (Fas^{lpr}) fibroblasts after treatment with mtFN (50 μg/ml) for 5 h. Bottom, percentage of apoptotic cells determined by flow cytometry after treatment with mtFN (50 μg/ml) for 5 h. (B) Top left, intracellular localization of GFP-Fas or GFP proteins after transfection with GFP-Fas or GFP vector controls (1.0 μg). Bars, 10 μm. Top right, phase-contrast images of Fas^{lpr} fibroblasts after transfection with GFP-Fas and treatment with mtFN (50 μg/ml) for 5 h. Bottom, percentage of apoptotic cells determined by flow cytometry after treatment with mtFN (50 μg/ml) for 5 h. (C) Fas^{lpr} fibroblasts were transfected with GFP-Fas cDNA and treated with mtFN (50 μg/ml) for 5 h. Lysates were immunoprecipitated and immunoblotted as indicated. (D) Fas^{lpr} cells were stably transfected with empty vector or Fas and resultant clones were designated as GFP-Fas^{lpr} and GFP-Fas-Fas^{lpr}, respectively. -Fold change of DNA fragmentation in control (GFP-Fas^{lpr}) and Fas expressing (GFP-Fas-Fas^{lpr}) clones after treated with mutant fibronectin (mtFN; 50 μg/ml) for 16 h. Inset, Fas overexpression was confirmed at the protein level using Western blot against GFP. β-Actin served as loading control. Bars, 50 μm for phase-contrast images. *, #$p \leq 0.01$.

RIP Is a Proapoptotic Transducer of Fas-mediated Anoikis

To further confirm the role of RIP in Fas-mediated anoikis, we tested wild-type and RIP-null ($RIP^{-/-}$) MEFs under anoikis conditions. Like SCC cells, the $RIP^{+/+}$ wild-type cells underwent anoikis; however, the $RIP^{-/-}$ cells were resistant to anoikis (Figure 3C). Forced expression of RIP in $RIP^{-/-}$ cells significantly increased anoikis (Figure 3D). These data indicate that RIP deficiency makes cells resistant to anoikis and that RIP is a proapoptotic mediator in Fas-mediated anoikis. Furthermore, silencing RIP with siRNA rescued SCC cells from anoikis, confirming that RIP is an important mediator in matrix-regulated apoptosis (Figure 3E, top and bottom).

RIP's Death Domain Is Critical to Mediating Anoikis and Shuttling between Fas and FAK

To identify the RIP domain that mediates anoikis, we tested RIP deletion mutants (Figure 4A) and examined their ability to modulate anoikis induced by mtFN in SCC and $RIP^{-/-}$ cells. Wild-type RIP and a RIP mutant containing only the death domain (aa 559-671; $RIP_{559\text{-}671}$) increased matrix-induced apoptosis equally, whereas a RIP mutant containing the kinase and intermediate domains (aa 1-558; $RIP_{1\text{-}558}$) did not induce anoikis beyond controls (Figure 4, B and C). RIP expression in transfected SCC and $RIP^{-/-}$ cells was individually optimized and confirmed by Western blot analysis (Figure 4, B and C). Together, these data indicate that RIP's death domain is sufficient and essential in matrix-induced cell death.

To identify the RIP domains that are critical for binding to FAK and Fas, and thus for RIP's shuttling function, we tested the ability of the RIP mutants to form complexes with Fas and FAK using coimmunoprecipitation experiments in transfected $RIP^{-/-}$ cells. Under anoikis conditions, cells expressing wild-type RIP and $RIP_{559\text{-}671}$ formed complexes with Fas to a greater extent than those expressing $RIP_{1\text{-}558}$ (Figure 4C). In addition, the lowest FAK and RIP complexes were formed in the absence of the RIP kinase domain, underscoring the critical role of this domain in FAK–RIP interactions. Together, these data demonstrate that RIP's death domain is the critical RIP region for complexing with Fas, whereas the kinase domain is the critical RIP region for complexing with FAK in regulating anoikis and shuttling between Fas and FAK. Based on these findings, we propose a model for RIP's dual role in regulating survival and anoikis signaling pathways (Figure 4D).

DISCUSSION

This study shows that RIP is a key signaling and shuttling protein that communicates with both integrin/FAK survival signals and Fas death signals in anoikis. Furthermore, although previous reports show that RIP interacts with FAK or Fas under separate conditions, our data reveal for the first time that RIP connects the two pathways. Thus, RIP could be an important early and upstream target for regulating anoikis in SCC and different cell systems. Treatment with mtFN, which alters the fibronectin matrix and creates conditions that lead to anoikis, induced FasL expression and subsequent Fas induction and activation by mediating the formation of death-inducing signaling complexes. Up-regu-

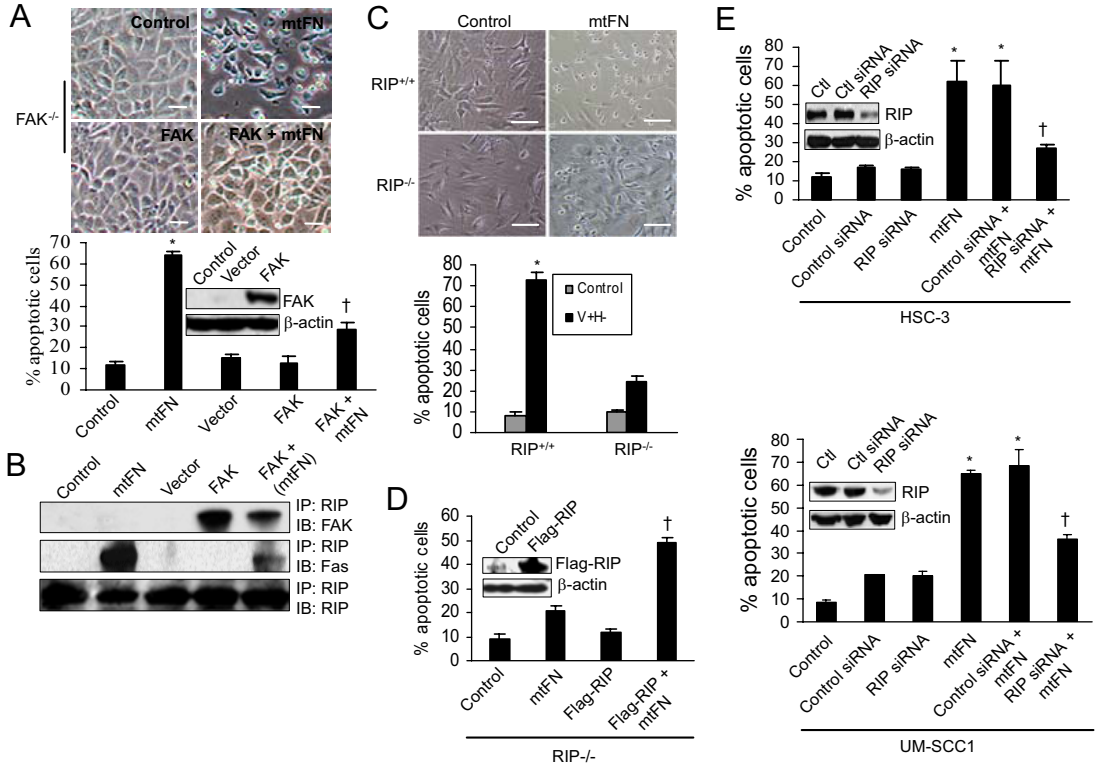

Figure 3. RIP shuttles between FAK and Fas to regulate anoikis. (A) Top, phase-contrast images of FAK$^{-/-}$ MEFs after treatment with mtFN (50 μg/ml) for 16 h. Bottom, percentage of apoptotic cells determined by flow cytometry after transfection with FAK (1.0 μg) or vector control and treatment with mtFN (50 μg/ml) for 16 h. Inset, immunoblots showing the FAK levels in cells after transfection with FAK (1.0 μg) or vector control. (B) FAK$^{-/-}$ MEFs were transfected with FAK cDNA and treated with mtFN (50 μg/ml) for 3 h. Lysates were immunoprecipitated and immunoblotted as indicated. (C) Top, phase-contrast images of wild-type (RIP$^{+/+}$) and RIP$^{-/-}$ MEFs after treatment with mtFN (50 μg/ml) for 5 h. Bottom, percentage of apoptotic cells determined by flow cytometry after treatment with mtFN (50 μg/ml) for 5 h. (D) Percentage of apoptotic cells in RIP$^{-/-}$ cells after transfection with FLAG-RIP (1.0 μg) and treated with mtFN (50 μg/ml) for 5 h. Inset, immunoblot showing RIP levels after transfection with FLAG-RIP (1.0 μg). (E) Percentage of apoptotic cells in SCC cells (top, HSC-3; bottom, UM-SCC1) determined by flow cytometry after transfection with RIP siRNA (50 nM) and treated with mtFN (50 μg/ml) for 16 h. Inset, immunoblot showing RIP levels after transfection with RIP siRNA (50 nM). Bars, 50 μm. *, #p $\leq$ 0.01.

lation of FasL and Fas and formation of the death-inducing signaling complex are key signals that initiate apoptosis by agents such as agonistic Fas antibody, cisplatin, and methotrexate in different cancer cells (Friesen *et al*., 1996; Uslu *et al*., 1997). The elevated levels of Fas and the formation of the death-inducing signaling complex under anoikis conditions, and the rescue of cells from anoikis by Fas siRNA treatment or Fas deficiency, further confirmed that anoikis is mediated by the Fas/CD95 death receptor pathway. These findings indicate that Fas activation and formation of the death-inducing signaling complex are critical for anoikis. Although DR5 induction was relatively low, the possibility of DR5 involvement in mediating anoikis is not ruled out.

Detachment of epithelial cells and SCC cells from their substratum or matrix leads to decreased integrin/FAK-mediated survival signaling, resulting in anoikis (Frisch and Francis, 1994; Zhang *et al*., 2004). In esophageal SCC cells, suppression of FAK promotes anoikis and suppresses metastasis (Duxbury *et al*., 2004), whereas overexpression of FAK correlates with tumor invasiveness and metastasis (Miyazaki *et al*., 2003). FAK in general transduces integrin-mediated signals that regulate survival and migration (Parsons, 2003).

Because decreased FAK survival signals and activation of Fas death signals were involved in matrix-induced apoptosis in SCC cells, we hypothesized that these two pathways communicate through a shuttling protein. Indeed, RIP functioned in that capacity. Under anoikis conditions, RIP dissociated from FAK and formed a complex with Fas, whereas under survival conditions, it dissociated from Fas and complexed with FAK. In FAK$^{-/-}$ cells, reintroduction of FAK promoted survival and allowed RIP to complex with FAK; under anoikis conditions, however, RIP-FAK complex levels were decreased. RIP$^{-/-}$ cells were resistant to anoikis, and reintroduction of RIP into these cells sensitized them to anoikis. RIP reintroduction also enabled the formation of RIP–FAK complexes; under anoikis conditions, those complexes were reduced, and RIP–Fas complexes became more abundant. These findings demonstrate that RIP shuttles between Fas and FAK to regulate anoikis.

In cell death pathways, RIP is activated and cleaved upon treatment with TNF, Fas, or TRAIL (Lin *et al*., 1999; Martinon *et al*., 2000). However, RIP can also participate in survival pathways (Meylan and Tschopp, 2005). RIP$^{-/-}$ cells are resistant to staurosporine- and cisplatin-induced apoptosis (Kurenova *et al*., 2004) but are sensitive to TNF-induced apoptosis (Cusson *et al*., 2002), suggesting a dual role for RIP in apoptosis. In this study, we showed that anoikis conditions trigger the dissociation of RIP from FAK and the formation of a RIP–Fas complex that initiates cell death, indicating that RIP plays a proapoptotic role in anoikis.

To further confirm that RIP acts as a proapoptotic or prosurvival mediator, we tested RIP$^{+/+}$ and RIP$^{-/-}$ fibro-

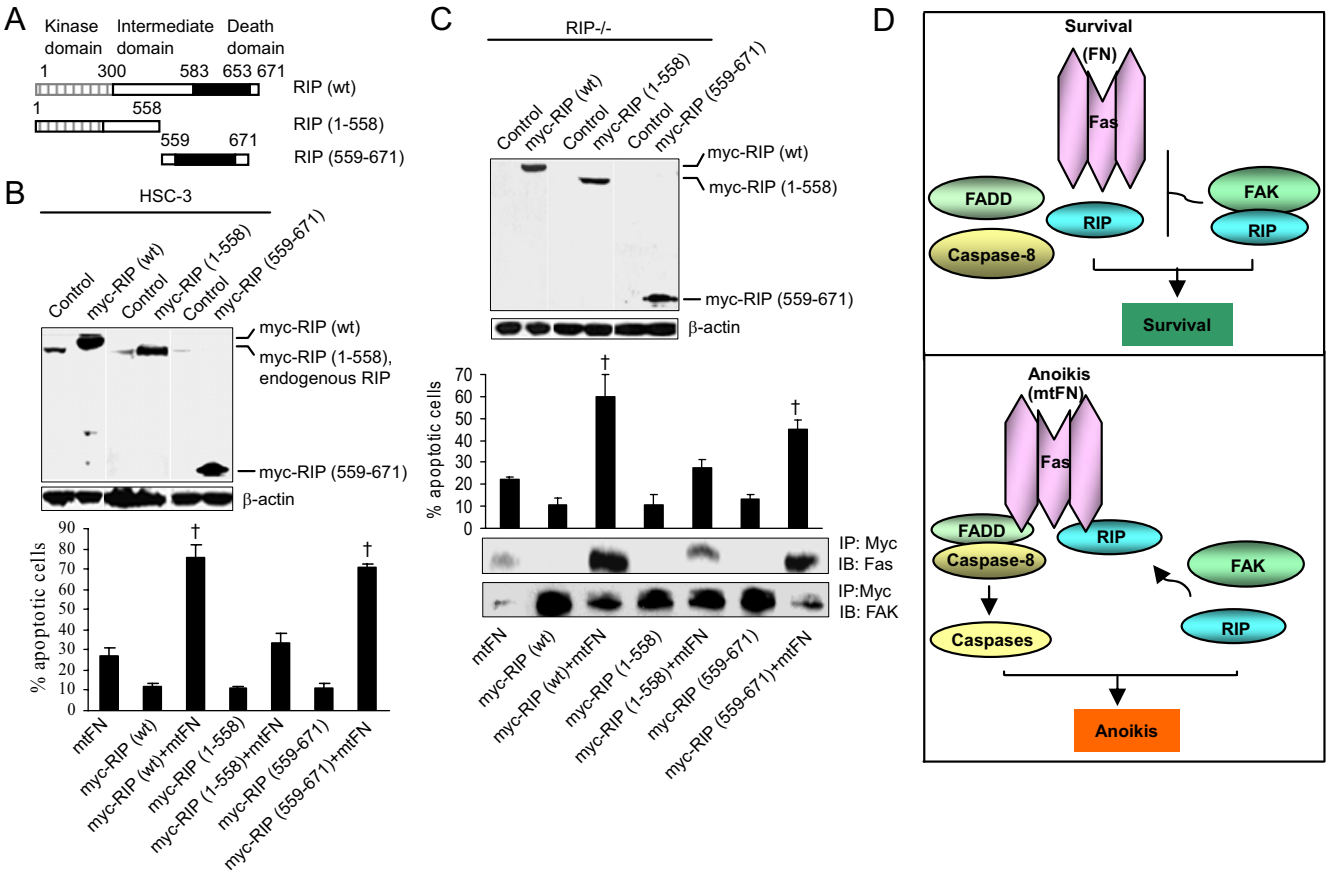

Figure 4. Functional mapping of RIP's apoptosis-inducing domain in SCC and RIP$^{-/-}$ cells. (A) Schematic diagram of wild-type RIP and RIP mutants. (B) Top, immunoblots showing RIP levels in SCC cells (HSC-3) after transfection with 1.5 μg of myc-RIP, myc-RIP$_{1-558}$, or myc-RIP$_{559-671}$. Bottom, percentage of apoptotic cells determined by flow cytometry after treatment with mtFN (25 μg/ml) for 16 h. (C) Top, Immunoblots showing RIP levels in RIP$^{-/-}$ cells after transfection with 1.5 μg of myc-RIP, myc-RIP$_{1-558}$, or my-RIP$_{559-671}$. Middle, percentage of apoptotic cells determined by flow cytometry after treatment with mtFN (50 μg/ml) for 16 h. Bottom, RIP$^{-/-}$ cells were transfected as in top panel and treated with mtFN (50 μg/ml) for 3 h. Lysates were immunoprecipitated with Myc and immunoblotted with Fas or FAK. (D) Model of the dual role of RIP in regulating anoikis and survival. In the presence of a prosurvival matrix (intact fibronectin; FN), FAK and RIP associate, thereby inhibiting the association of RIP with Fas and formation of the death-inducing signaling complex (Fas, FADD, and caspase-8) and leading to cell survival through activation of cell survival signals, such as phosphorylation of FAK and ERK. In the presence of a proapoptotic matrix (mtFN), FAK and RIP dissociate, leading to the association of RIP with Fas and formation of the death-inducing signaling complex. In parallel, caspase-8 may induce activation of RIP cleavage and activation of other downstream effector caspases, leading to cell death by anoikis. #p $\leq$ 0.01.

blasts under anoikis conditions. RIP$^{-/-}$ cells were resistant to anoikis; however, reintroduction of RIP rendered them sensitive to anoikis. Thus, RIP is a proapoptotic mediator essential for anoikis. To further demonstrate that RIP also regulates cell survival pathways, we tested its role in FAK$^{-/-}$ MEFs, which are highly sensitive to RIP-mediated apoptosis. Ectopic expression of FAK restored the interaction of RIP with FAK and the concomitant dissociation of RIP from Fas, thereby rescuing cells from anoikis and confirming that RIP interacts with both death and survival signals. Thus, FAK overexpression in SCC cells prevents the interaction of RIP with Fas, making cells resistant to death receptor-induced apoptosis. Given its ability to mediate both cell death and cell survival pathways, RIP could be a potential therapeutic target for SCC.

RIP cleavage leads to the inhibition of the NF-κB pathway, thereby potentiating apoptosis (Lin *et al.*, 1999). Therefore, we hypothesize that NF-κB acts downstream of RIP in anoikis. Consistent with these findings, rituximab sensitizes cells to apoptosis by increasing Fas expression and inhibiting the NF-κB signaling pathway (Vega *et al.*, 2005). Thus, under anoikis conditions, RIP cleavage may inhibit NF-κB activity to potentiate apoptosis.

The RIP death domain is important for TNF-induced cell death (Thakar *et al.*, 2006), whereas the kinase domain is important for survival signals such as those mediated by ERK (Devin *et al.*, 2003) and the epidermal growth factor receptor (Habib *et al.*, 2001). Consistent with this finding, we showed that the RIP deletion mutant containing the death domain is sufficient to induce anoikis, whereas the RIP mutant containing the kinase and intermediate domains did not enhance anoikis. Also, the RIP kinase domain mutant formed the lowest level of complexes with FAK, indicating the importance of the kinase domain in FAK interactions. Thus, the RIP death domain is sufficient and essential for anoikis in SCC cells.

Based on our findings and published literature, we propose that RIP regulates anoikis by shuttling between the CD95/Fas-mediated death pathway and the integrin/FAK-mediated survival pathway (Figure 4D). In the presence of a prosurvival matrix (i.e., intact fibronectin), FAK and RIP associate, thereby inhibiting the association of RIP with Fas

and leading to cell survival through phosphorylation of FAK and activation of cell survival signals, such as ERK. Under anoikis conditions, FAK and RIP dissociate, leading to the association of RIP with Fas and the formation of the death-inducing signaling complex. Caspase-8 activates downstream effector caspases, and it may also induce RIP cleavage leading to cell death. Cleaved RIP may inhibit or neutralize NF-κB and its downstream survival signals. In addition, inhibition of integrin/FAK signals probably leads to reduced ERK phosphorylation and further loss of survival signals.

In conclusion, our findings show that RIP is required for anoikis induced by the Fas/CD95 death receptor, and RIP shuttles between Fas and FAK to mediate this mechanism. Our report supports the notion that therapeutic pharmaceuticals can target RIP as a switch to control cell death or survival pathways to ultimately regulate normal tissue processes and tumorigenesis.

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