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Focal Adhesion Kinase Suppresses Apoptosis by Binding to the Death Domain of Receptor-Interacting Protein

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Tumor cells resist the apoptotic stimuli associated with invasion and metastasis by activating survival signals that suppress apoptosis. Focal adhesion kinase (FAK), a tyrosine kinase that is overexpressed in a variety of human tumors, mediates one of these survival signals. Attenuation of FAK expression in tumor cells results in apoptosis that is mediated by caspase 8- and FADD-dependent pathways, suggesting that death receptor pathways are involved in the process. Here, we report a functional link between FAK and death receptors. We have demonstrated that FAK binds to the death domain kinase receptor-interacting protein (RIP). RIP is a major component of the death receptor complex and has been shown to interact with Fas and tumor necrosis factor receptor 1 through its binding to adapter proteins. We have shown that RIP provides proapoptotic signals that are suppressed by its binding to FAK. We thus propose that FAK overexpression in human tumors provides a survival signal function by binding to RIP and inhibiting its interaction with the death receptor complex.

Focal adhesion kinase (FAK) is a nonreceptor protein tyrosine kinase that plays a key role in maintaining focal adhesion function and cell survival and is implicated in cell migration, adhesion, and cell cycle control (9, 13, 18, 20, 33, 44). Overexpression of FAK is a common event in numerous tumor systems, including breast, colon, and thyroid carcinomas (2, 24, 32, 41), and occurs at early stages of tumorigenesis, before a tumor has developed the capacity for invasion and metastasis (2). Importantly, FAK has been shown to be one of the critical factors protecting cells from apoptosis, but the exact mechanism is unknown (8, 9, 12, 19, 37, 43). Attenuation of FAK expression by antisense oligonucleotides led to apoptosis in tumor cells (42), and the treatment of cells with anti-FAK antibody (18, 26) or overexpression of the focal adhesion targeting (FAT) domain of FAK led to cell rounding, detachment, and apoptosis (19, 21, 40). We have created a model system for the attenuation of FAK function by adenoviral gene transduction of the carboxy-terminal domain of FAK (FAK-CD) and have demonstrated a loss of adhesion and apoptosis in breast cancer cells with this treatment (43). Both anchorage-dependent and anchorage-independent apoptotic signaling required Fas-associated death domain protein (FADD) and caspase 8, suggesting an important role for FAK in inhibiting death receptor-related apoptosis (43). This finding provided additional evidence that a death receptor-mediated apoptotic pathway or death receptor-related death domain proteins are involved in the apoptotic process triggered by the expression of FAK-CD.

The loss of adhesion and induction of apoptosis upon attenuation of FAK function by the expression of FAK-CD is similar to the phenomenon of anoikis (7–9). Intriguingly, there is evidence for the involvement of death receptor-related, death domain-containing proteins in anoikis (7, 35), whereby the silencer of death domain (SODD) and dominant-negative FADD efficiently inhibited anoikis in Madin-Darby canine kidney (MDCK) cells and in a number of untransformed epithelial cell lines. In these studies, it was also shown that cell matrix detachment activated caspase 8. However, the linkage of the signaling pathways to the death receptors remains unknown.

RIP is a serine/threonine kinase that contains a death domain (17, 38) and is named for its association with the death receptor complex. RIP interacts with the death domains of cell surface receptors of the tumor necrosis factor (TNF) superfamily and death domain adaptor proteins (3, 5, 17) and plays an indispensable role in NF-κB activation (23, 39). Recently, it was shown that TNF alpha-mediated activation of NF-κB depends on the association of RIP and FAK (11). TNF-induced NF-κB DNA binding activity and activation of IκB kinases were markedly impaired in $FAK^{-/-}$ cells (11). However, it has been well established that RIP has a dual function and is capable of either inducing apoptosis or activating cellular survival signals (14, 17, 23, 27, 38, 39). Similarly, it has been proposed that RIP is one of the switches between cell survival and apoptosis (28). However, the mechanisms by which such diverse functions are selected remain unclear.

To elucidate the possible protein(s) involved in apoptosis

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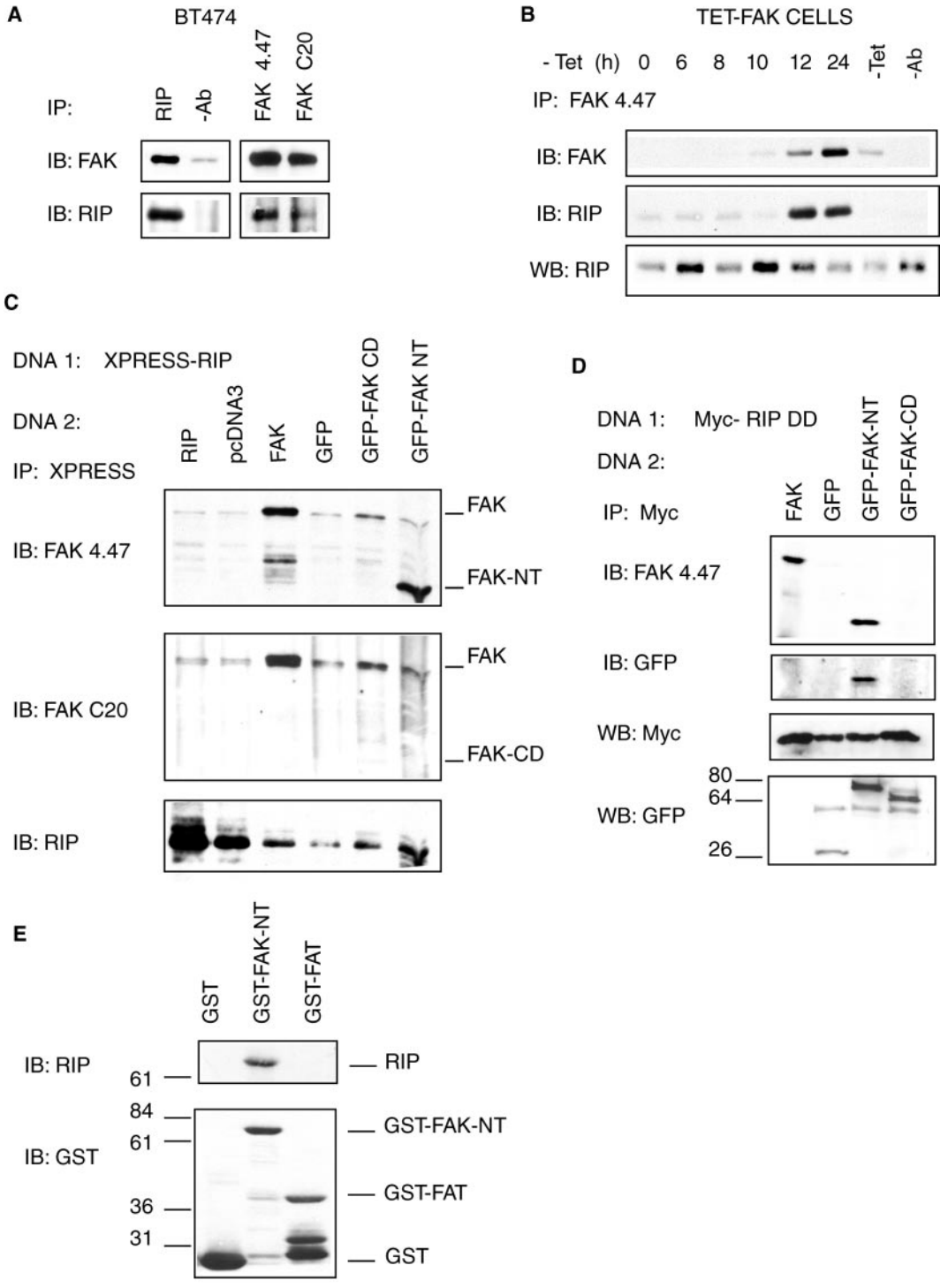


FIG. 1. Association between FAK and RIP in vivo and in vitro. (A) Human breast carcinoma cell line BT-474 was grown in monolayer. Binding was analyzed by immunoprecipitation with anti-RIP antibody and then followed by immunoblotting with anti-FAK antibody or by immunoprecipitation with anti-FAK antibody against N terminus 4.47 or C terminus C20 followed by IB with anti-RIP antibody. Probe without antibody was added as a control for background binding. (B) Tet-FAK cell lysates were immunoprecipitated with anti-FAK antibody followed by immunoblotting with anti-RIP antibody. A Western blot of Tet-FAK cell lysates with RIP antibody was performed to evaluate RIP synthesis in the cells during the time course of tetracycline withdrawal. Probe without antibody was added as a control for background binding. (C) HEK293 cells were transiently cotransfected with pXpress-RIP along with pcDNA3, pcDNA3-FAK, pGFP C1 vector, pGFP-FAK-CD, or pGFP-FAK-NT. pCMV-CrmA was also included in the transfections to protect the cells from apoptosis induced by the overexpression of RIP. After 24 h, cell lysates were subjected to immunoprecipitation with anti-Xpress antibody. The immunopellets were analyzed by immunoblotting and probing with anti-FAK

caused by the disruption of FAK signaling by expression of the C-terminal domain of FAK, we tested several death domain proteins for the ability to bind to FAK. We found that RIP bound to FAK in vitro and in vivo in different mammalian cell lines (normal and transformed), and the level of apoptosis caused by the attenuation of FAK correlated with the level of RIP expression in the particular cell line. In this report, we show a possible mechanism by which the death receptor machinery is involved in anoikis through the interaction of FAK and RIP.

MATERIALS AND METHODS

Cell culture. BT-474 and BT-20 human breast carcinoma cell lines were cultured as described previously (43). Tet-FAK cells, whereby the tetracycline repression system was implemented in FAK-null mouse embryonic fibroblasts to modulate FAK expression, were cultured as described previously (31). RIP$^{-/-}$ TNFR1$^{-/-}$ and RIP$^{+/+}$ TNFR1$^{-/-}$ mouse embryonic fibroblasts (4) were kindly provided by P. Leder and M. Kelliher (Harvard Medical School, Boston, Mass.). HEK293 human embryonic kidney cells were cultured in Dulbecco modified Eagle medium containing 10% fetal bovine serum. All cell lines were incubated at 37°C in 5% CO_2.

Plasmids, reagents, and antibodies. The mammalian expression vector encoding hemagglutinin (HA)-FAK has been described previously (43). pXpress-RIP and pMyc-RIP death domain were kindly provided by Zheng-gang Liu (National Cancer Institute, National Institutes of Health, Bethesda, Md.). The glutathione transferase (GST)-RIP construct was prepared by subcloning RIP into the SmaI site of the pGEX-3X vector. The FAT sequence (nucleotides 2987 to 3391 of FAK) was subcloned by PCR into the pGEX-4T-1 vector. GST-FAK-N terminus (NT), green fluorescent protein (GFP)–FAK-NT, and GFP-FAK-CD were previously described (1). Staurosporine (Calbiochem) was used in 200 and 500 nM concentrations. We used anti-FAK 4.47 (Upstate Biotechnology, Inc.) monoclonal antibody and C20 (Santa Cruz Biotechnology) polyclonal antibody; anti-HA antibody (12CA5, Roche Molecular Biochemicals) and anti-RIP monoclonal antibody (Transduction Laboratory); anti-FAK phosphotyrosine Y397 antibody (Biosourse International, Inc); and monoclonal anti-β-actin and anti-GST antibody (Sigma), anti-Xpress antibody and anti-Myc antibody (Invitrogen), and anti-GFP antibody (Clontech).

Transfection. Cells were plated at a density of 2×10^6 cells per 100-mm culture plate or 2×10^5 cells per well in six-well plates and allowed to attach for 24 h and then were transfected by using Lipofectamine (Invitrogen) for BT-474 and BT-20 cells and Effectene (QIAGEN) for HEK293 cells according to the manufacturer's instructions.

Immunoprecipitation and Western blotting. For cell lysate preparation, 1% NP-40 lysis buffer was used (20 mM Tris-HCl [pH 8.0], 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin/ml, 20 μg of leupeptin/ml). The protein concentration of lysates was determined by using the bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.). For immunoprecipitation, lysates (500 μg of total protein) were precleared with protein A/G-agarose beads (Oncogene Research Products Inc.) at 4°C for 1 h and then incubated with 5 μl of antibody for 2 h followed by overnight incubation with protein A/G-agarose beads at 4°C. Precipitates were washed three times in lysis buffer, and beads were resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer, boiled for 5 min, and resolved by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane, probed with appropriate antibody, and detected by chemiluminescence (KPL, Gaithersburg, Md.).

In vitro binding. GST fusion proteins were produced and purified as previously described (43). For in vitro binding assay, cell lysates were precleared with GST protein (5 μg), and the cleared supernatants (500 μg) were incubated for 1 h at 4°C with 1, 5, or 10 μg of GST fusion protein immobilized on glutathione-agarose beads. Cell lysates were prepared from different cell lines or from 293 cells transfected with expression vectors encoding RIP, FAK, or its fragments. The beads were washed twice with lysis buffer and twice with phosphate-buffered saline (PBS). The bound proteins were analyzed by Western blotting.

Adenoviral infections. Adenovirus (Ad) FAK-CD (amino acids 693 to 1052 of FAK fused to the HA epitope tag) was described previously (43). The Ad carrying the *lacZ* gene was provided by J. Samulski (University of North Carolina, Chapel Hill, N.C.). Cells were plated at a density of 2×10^6 cells per 100-mm culture plate or 2×10^5 cells per well in six-well plates, allowed to attach for 24 h, and then infected with Ad FAK-CD or Ad LacZ for various times in complete medium. We typically infected cells with 200 focus-forming units (FFU)/cell, and expression was verified by Western blotting with an anti-HA antibody that allowed us to estimate that infectivity and expression of Ad were equal in all types of cells used.

Immunofluorescence staining. Cells were incubated in the presence or absence of staurosporine or Ad and stained with anti-FAK monoclonal antibody 4.47 as previously described (43).

siRNA synthesis and transfection. Small interfering RNAs (siRNAs) were prepared according to the Silencer siRNA construction kit protocol (Ambion). RIP siRNA H87 corresponded to the coding region 1129-1149 relative to the first nucleotide of the start codon, and the RIP mRNA target sequence is 5′-ACTC CAAGACGAAGCCAAC-3′. siRNA H121 corresponded to the coding region 1693-1713, and the RIP mRNA target sequence is 5′-AGAAGAGCCAGCTG CTAAG-3′. siRNA H121Scr is a scrambled sequence of H121 siRNA. siRNA GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was synthesized according to the Silencer kit protocol (Ambion). The transfection of siRNAs to target the endogenous genes was carried out by using Oligofectamine (Invitrogen) according to the manufacturer's protocol.

PolyHEMA-coated tissue culture dishes. Tissue culture dishes were coated with a film of poly(2-hydroxyethylmethacrylate) (polyHEMA; Sigma) as described by Folkman and Moscona (6). Cells were trypsinized, resuspended, and added to the plates coated with polyHEMA at a density of 2×10^6 cells per dish in a serum-containing medium. Cells were maintained in suspension culture for various times and assayed for cell survival by methods such as terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) and methyl thiazole tetrazolium (MTT) assays.

Assays of cell viability. Detection of apoptosis was performed by TUNEL assay and Hoechst 33342 staining. Cells were collected by centrifugation, fixed in 3.7% formaldehyde in 1× PBS for 10 min, washed twice with 1× PBS, and stained with Hoechst 33342 or spread on the slide for TUNEL staining. TUNEL assay was done with the ApopTag kit (Intergen, Purchase, N.Y.) according to the manufacturer's protocol. The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to the total number of cells counted with a fluorescence microscope in several fields of three independent experiments. Simultaneous staining and quantification of apoptotic cells with the Hoechst 33342 and TUNEL methods produced very similar results. Cell survival was assayed by measuring mitochondrial dehydrogenase activity, the conversion of soluble MTT into insoluble formasan product, as described by Mosmant (29).

RESULTS AND DISCUSSION

Association between FAK and RIP in vivo and in vitro. To determine whether there is a direct interaction between FAK and RIP, we tested their binding in breast cancer cell lines. Lysates were prepared from the BT-474 and BT-20 cell lines, and coimmunoprecipitation experiments were performed with antibody against FAK and RIP. We found a strong association

4.47 and C20 antibody and anti-RIP antibody. The expression of all constructs was confirmed with the corresponding antibody (data not shown). (D) HEK293 cells were transiently cotransfected with pcDNA-Myc-RIP death domain along with pcDNA3-FAK, pGFP C1 vector, pGFP-FAK-NT, or pGFP-FAK-CD. pCMV-CrmA was also included in the transfections. After 24 h, cell lysates were subjected to immunoprecipitation with anti-Myc antibody. The immunopellets were analyzed by immunoblotting and probing with anti-FAK 4.47 and C20 antibody (data not shown) and anti-GFP antibody. The expression of all constructs was confirmed by Western blot with the corresponding antibody (anti-Myc and anti-GFP antibody). (E) Association of endogenous RIP with GST-FAK-NT fusion protein. GST protein and GST-fused FAK fragments (NT or FAT domain) were bound to glutathione-Sepharose beads and used to pull RIP down from extracts of HEK293 cells, followed by SDS–10% PAGE. The immunodetection first used anti-RIP antibody to detect pulled-down RIP, and then the blot was reprobed with anti-GST antibody. IP, immunoprecipitation; IB, immunoblotting; −Ab, probe without antibody; WB, Western blot.

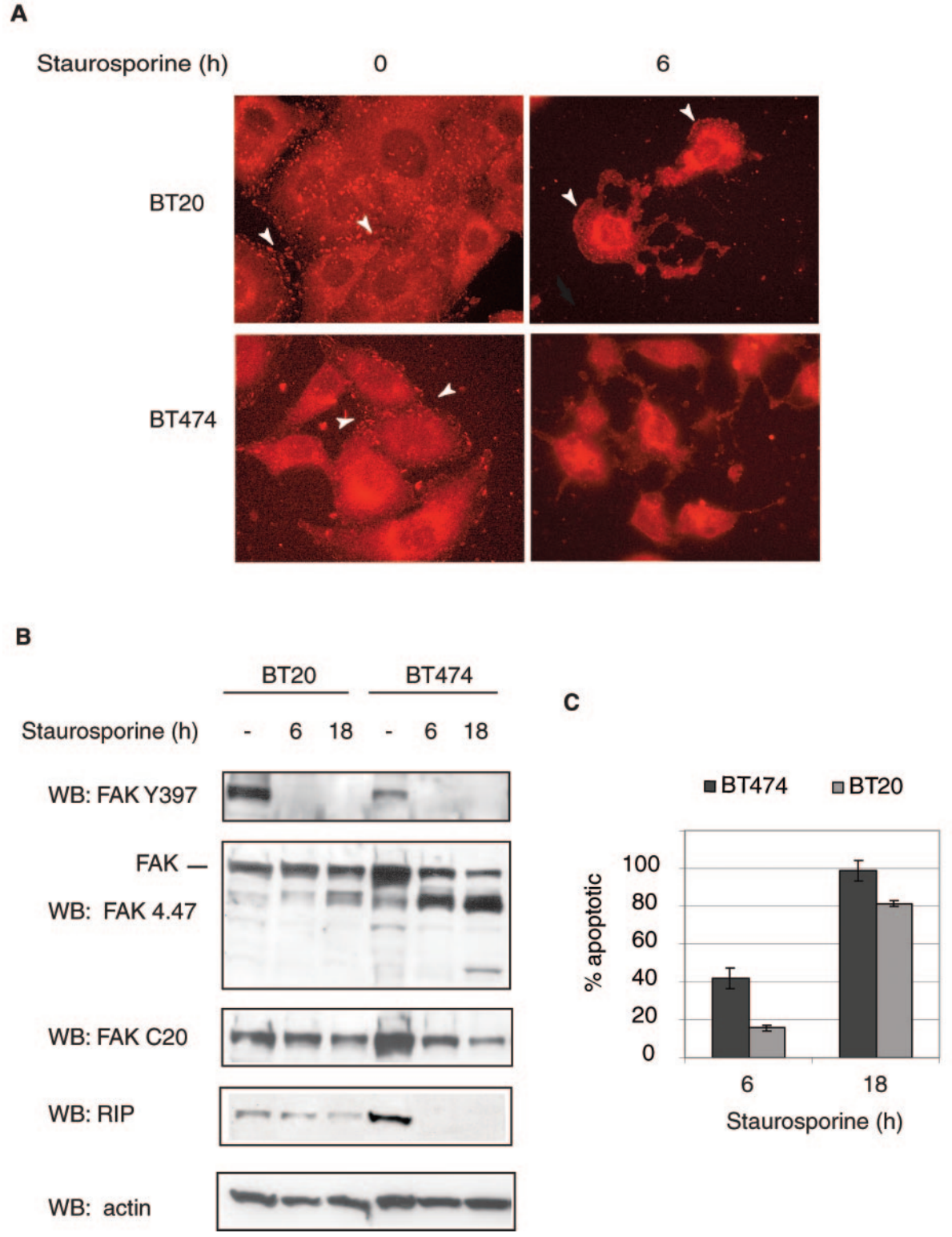


FIG. 2. Staurosporine causes dephosphorylation and degradation of FAK in breast carcinoma cell lines. A total of 2×10^5 cells from BT-20 or BT-474 cell culture were plated on six-well plates and were treated with staurosporine (200 nM) after 24 h. After 6 and 18 h of treatment, cells were immunostained with anti-FAK 4.47 antibody, detached cells were counted, and cells were collected for TUNEL assay and Western blot analysis. (A) Immunostaining of untreated cells and cells treated with staurosporine for 6 h (white arrowheads mark some focal adhesions). (B) Western blot (WB) analysis of BT-20 and BT-474 lysates treated with staurosporine. Control cells were treated with dimethyl sulfoxide for 18 h. (C) TUNEL assay data from one of three representative experiments.

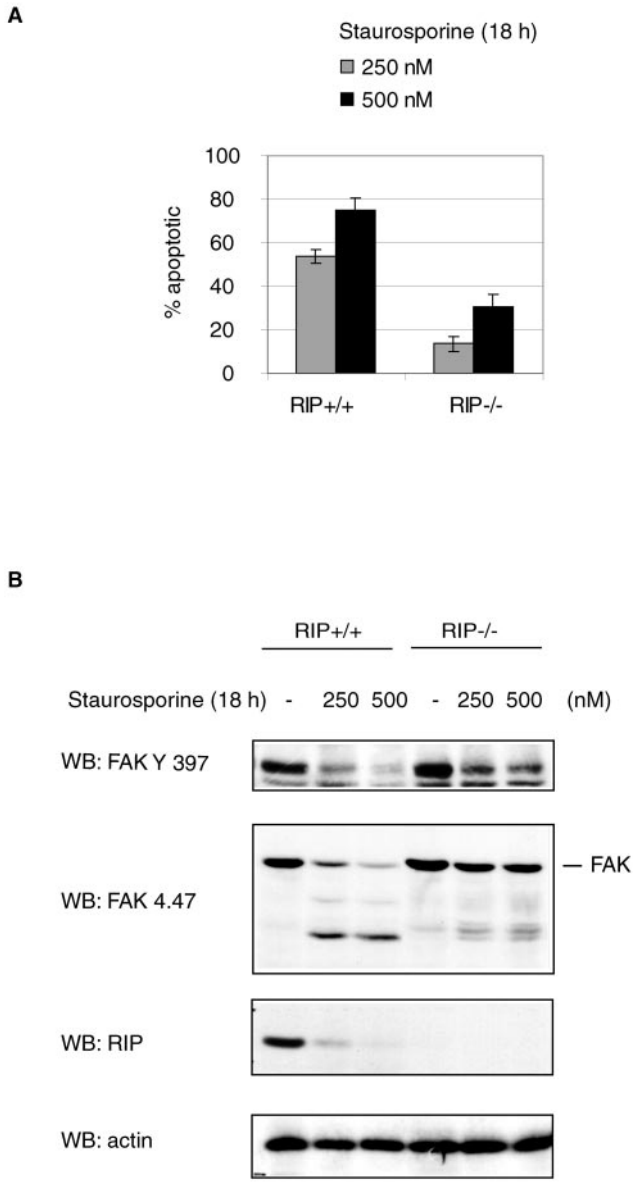


FIG. 3. Staurosporine causes dephosphorylation and degradation of FAK, which depends on the presence of RIP. A total of 2×10^5 cells from $RIP^{+/+}$ or $RIP^{-/-}$ cell culture were plated on six-well plates and were treated with staurosporine after 24 h. After 18 h of treatment, detached cells were counted, and cells were collected for TUNEL assay and Western blot analysis. (A) TUNEL assay data from one of three representative experiments. (B) Western blot (WB) analysis of $RIP^{+/+}$ and $RIP^{-/-}$ lysates treated with staurosporine.

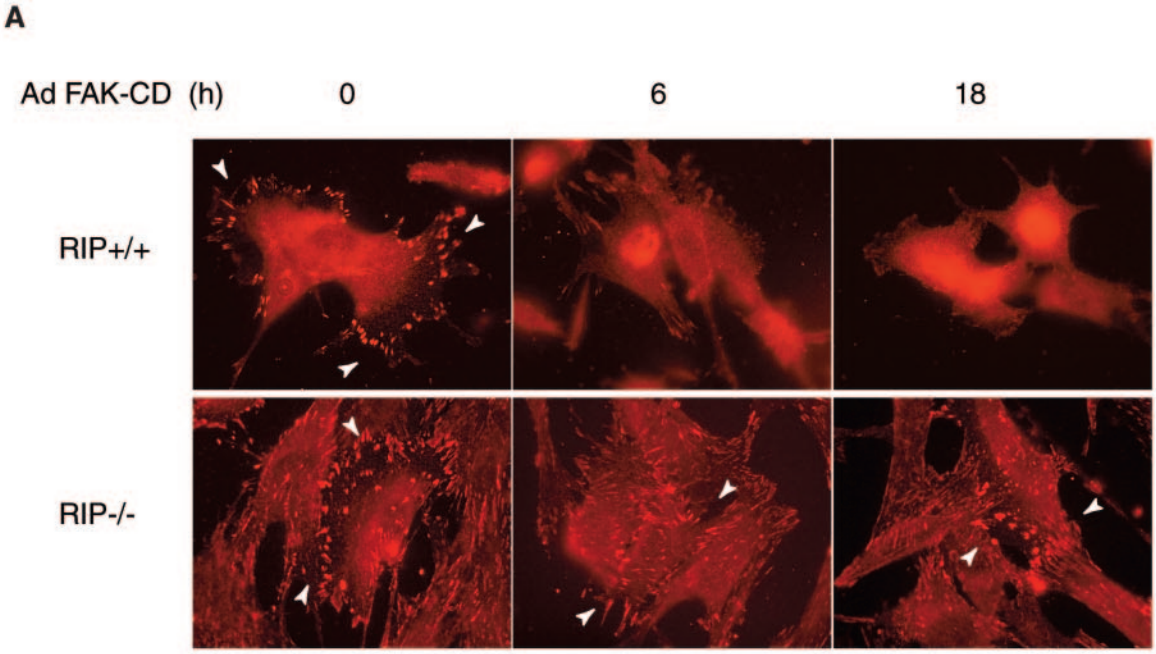
of FAK and RIP in both the BT-474 (Fig. 1A) and the BT-20 (data not shown) cell lines. To confirm the specificity of this binding, we used an inducible system of FAK expression (Tet-FAK cells) in FAK-null mouse embryonic fibroblasts, where FAK expression is under the control of a tetracycline repressor (31). Between 12 and 24 h after tetracycline withdrawal, cells expressed high levels of FAK (Fig. 1B). Immunoprecipitation of FAK from Tet-FAK cells demonstrated the association of FAK and RIP, with the level of coimmunoprecipitation proportional to the amount of FAK protein expression upon the withdrawal of tetracycline from the medium. Taken together, these results show that FAK and RIP bind together in both breast cancer cell lines and mouse embryonic fibroblasts.

To define the RIP-binding domain in FAK as well as the FAK-binding domain in RIP, we coexpressed different RIP and FAK plasmids in HEK293 cells. Full-length RIP bound to full-length FAK in HEK293 cells, and this binding appeared to occur at the amino terminus of FAK, since antibody against the C terminus of FAK (C20) did not reveal the FAK-CD protein in immunoprecipitates (Fig. 1C). When we expressed the death domain of RIP with different FAK constructs, RIP again bound both to full-length FAK and to the amino terminus of FAK (GFP-FAK-NT; Fig. 1D). To confirm this binding in vitro, we used GST-FAK fusion proteins to test their ability to bind to endogenous RIP in HEK293 cells. As shown in Fig. 1E, RIP bound only to the amino terminus of FAK (GST-FAK-NT), and did not bind to the focal adhesion targeting sequence in the C terminus of FAK (GST-FAT) or to control GST. From these experiments, we conclude that RIP binds to the amino terminus of FAK through the death domain both in vivo and in vitro.

Staurosporine-induced apoptosis causes RIP-dependent dephosphorylation and degradation of FAK. Next, we sought to determine the biological significance of the FAK-RIP interaction. First, we tested staurosporine, a bacterial alkaloid with broad inhibitory activities against a variety of serine/threonine kinases (34) and several tyrosine kinases (10, 30, 36). It also induces apoptosis in a variety of transformed cell lines and has been associated with FAK-mediated apoptosis in endothelial cells (22). In breast cancer cells, we showed that staurosporine caused a displacement of FAK from focal adhesions (Fig. 2A) and led to rapid dephosphorylation and slower degradation of FAK (Fig. 2B). We also found a decrease in the amount of RIP protein associated with FAK in BT-474 cells 2 to 3 h after treatment with staurosporine (data not shown), consistent with disruption of the interaction of FAK and RIP.

In parallel with the degradation of FAK, we observed an attenuation of RIP expression, resembling the proteosomal degradation of RIP, that has been described during the disruption of the RIP-Hsp90 complex with geldanamycin (25). Staurosporine induced different rates of apoptosis in these two cell lines (Fig. 2C), and intriguingly, the apoptotic rate was higher for the BT-474 cells that expressed significantly more RIP than that for the BT-20 cells (Fig. 2B). Based on densitometry analysis (data not shown), we estimated that BT-474 cells express 60% more RIP, suggesting that RIP may be providing proapoptotic signals in these breast cancer cells.

To specifically address this question, we tested staurosporine on embryonic fibroblasts derived from normal and $RIP^{-/-}$ mice (4). Staurosporine induced a higher rate of apoptosis in the $RIP^{+/+}$ cells than in the $RIP^{-/-}$ cells (Fig. 3A). Furthermore, staurosporine caused a dose-dependent dephosphorylation and degradation of FAK in the $RIP^{+/+}$ cells that was only minimal in the $RIP^{-/-}$ cells (Fig. 3B). The time course analysis of staurosporine treated $RIP^{+/+}$ and $RIP^{-/-}$ mouse fibroblasts by immunostaining and Western blot analysis showed that after 6 h of treatment, there were no focal adhesions in the rare, attached $RIP^{+/+}$ cells, while there were many attached $RIP^{-/-}$ cells with visible focal adhesion structures (data not shown). In


A
Ad FAK-CD (h)
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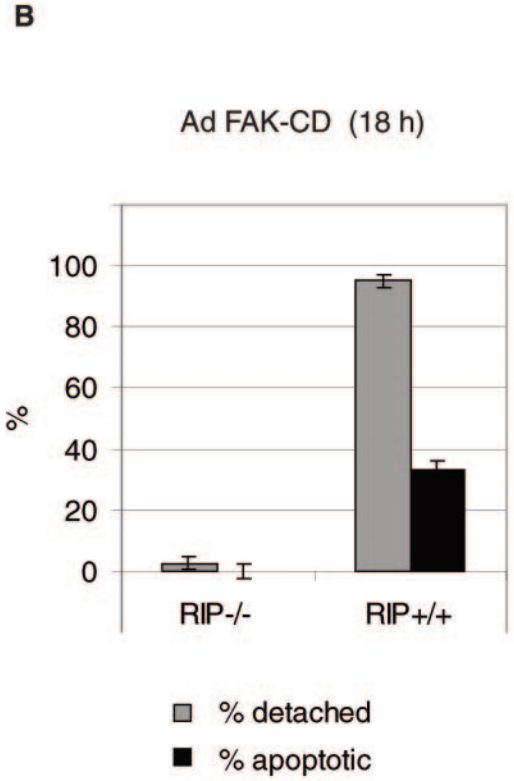
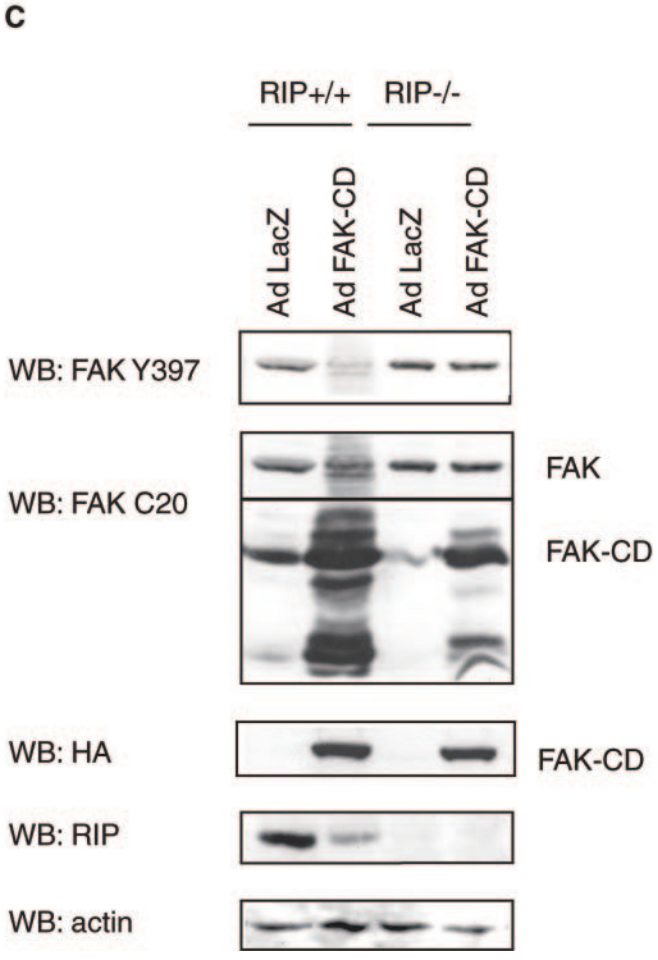
6
18
RIP+/+
RIP-/-


B
Ad FAK-CD (18 h)
100
80
60
40
20
0
%
RIP-/-
RIP+/+
% detached
% apoptotic


C
RIP+/+
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Ad LacZ
Ad FAK-CD
Ad LacZ
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WB: FAK Y397
WB: FAK C20
FAK
FAK-CD
WB: HA
FAK-CD
WB: RIP
WB: actin

addition, $RIP^{-/-}$ cells were resistant to FAK dephosphorylation and degradation, while FAK in $RIP^{+/+}$ cells was rapidly dephosphorylated and degraded (data not shown). These results show that the presence of RIP enhanced the rate of staurosporine-induced apoptosis and the level of FAK dephosphorylation and degradation, suggesting that RIP provides proapoptotic signals in these cells as well.

RIP is involved in transducing the proapoptotic signals initiated by the disruption of FAK signaling. To further define the relationship between RIP and FAK, we studied apoptosis that was induced by specific attenuation of FAK. We used our model system in which the transduction of an adenoviral FAK-CD construct into cells causes tyrosine dephosphorylation of FAK and displacement of FAK from the focal adhesions. In breast cancer cell lines, this subsequently led to detachment and apoptotic cell death, while in normal mammary epithelial cells, the displacement of FAK appeared to have no deleterious effect on the cells (43). Then, we applied this model to test the effect of FAK attenuation on the $RIP^{-/-}$ and $RIP^{+/+}$ fibroblasts. As shown in Fig. 4A, treatment of $RIP^{-/-}$ cells with Ad FAK-CD did not lead to the displacement of FAK from focal adhesions, while $RIP^{+/+}$ cells showed displacement of FAK beginning 6 h after treatment. In addition, $RIP^{-/-}$ cells did not detach from their substratum and had a very low level of apoptosis (Fig. 4B). Furthermore, $RIP^{-/-}$ cells had no significant changes in their level of FAK tyrosine phosphorylation and did not have significant degradation of FAK (Fig. 4C). In contrast, the $RIP^{+/+}$ cells detached from their substratum and underwent apoptosis (Fig. 4B) with corresponding changes in tyrosine phosphorylation and a resultant degradation of FAK (Fig. 4C). From these data, we conclude that the presence of FAK in the focal adhesions provides antiapoptotic signals to the cells, and once FAK has been displaced from focal adhesions, proapoptotic signals from RIP promote apoptosis and degradation of FAK.

RIP is involved in transducing the detachment-induced apoptotic signals. Different groups have shown that FAK controls adhesion-dependent cell survival in normal epithelial and endothelial cells (9, 12, 16, 19). Results from previous studies suggest that in breast tumor cells, FAK has two separate functions, one promoting the adhesive interactions between tumor cells and their extracellular matrix and the other acting as a survival signal that is independent of cellular adhesion (43). Thus, we compared the viability of $RIP^{+/+}$ and $RIP^{-/-}$ cells in the absence of matrix attachment. Cells were maintained on polyHEMA-coated tissue culture plates that create anchorage-independent conditions. Both $RIP^{+/+}$ and $RIP^{-/-}$ cells remained viable for at least 72 h, as determined by mitochondrial dehydrogenase activity using the MTT assay (data not shown). However, the viability of the suspended $RIP^{+/+}$ cells was lower than the viability of attached cells, with an average of 17% of the cells undergoing apoptosis after 72 h in anchorage-independent conditions (Fig. 5A) compared to 2% for the $RIP^{-/-}$ cells (Fig. 5A). This result suggests that RIP may be involved in transducing proapoptotic signals in anoikis. To examine whether protection from anoikis required FAK, we attenuated FAK with Ad FAK-CD. When $RIP^{-/-}$ cells grown in suspension were transduced with Ad FAK-CD, their rate of apoptosis was not different from $RIP^{-/-}$ cells transduced with Ad LacZ (Fig. 5B). In contrast, $RIP^{+/+}$ cells had a much higher level of apoptosis and became apoptotic much faster than $RIP^{-/-}$ cells when FAK signaling was disrupted (Fig. 5B). Thus, $RIP^{+/+}$ cells were still sensitive to FAK attenuation under anchorage-independent conditions. Taken together, these results demonstrate that RIP provides proapoptotic signals in fibroblasts grown in the absence of adhesion and that FAK plays an additional role as an adhesion-independent survival signal not only in transformed cells but also in normal fibroblasts.

Downregulation of RIP with siRNA in breast cancer cells leads to higher resistance to apoptosis caused by the disruption of FAK signaling. While these results have provided evidence of a proapoptotic role for RIP, we returned to our breast cancer cell model to determine whether the downregulation of RIP expression in BT-474 breast cancer cells could rescue these cells from apoptosis induced by FAK attenuation. To downregulate RIP expression, we synthesized two pairs of silencing siRNAs corresponding to human RIP (H87 and H121), which caused a moderate decrease in RIP expression in the BT-474 cells (Fig. 6A). After 48 h of siRNA transfection, cells were treated with either staurosporine (Fig. 6B and C) or FAK-CD (Fig. 6D and E). In the cells treated with staurosporine, a decrease in RIP expression by both RIP siRNAs protected FAK from rapid dephosphorylation during the first hour of treatment, while control siRNAs (H121Scr and GAPDH) had no effect. Longer treatment with staurosporine caused apoptosis, the level of which was lower in the cells treated with RIP siRNA (Fig. 6C). In the cells where FAK was specifically downregulated by FAK-CD, the reduction of RIP expression by siRNA also preserved a higher level of FAK phosphorylation even after a long exposure to FAK-CD. In contrast, FAK was dephosphorylated in untreated control cells (Fig. 6D). Furthermore, the rate of apoptosis (Fig. 6E) decreased from 42% in control cells to 22 and 15% in the H87 and H121 siRNA-treated cells, respectively. These results suggest that increased levels of FAK expression, accompanied by decreased levels of RIP expression, enhance the survival signals in breast cancer cells and make them more resistant to apoptotic stimuli.

These results provide a possible mechanistic explanation for the prosurvival function of FAK in human breast cancer. FAK binds to RIP in both normal and transformed cells, and the disruption of the balance between these two kinases in a cell may result in amplification of pro- or antiapoptotic stimuli. This result is consistent with other reports, where it has been

FIG. 4. RIP is involved in transducing the proapoptotic signals initiated by the disruption of FAK signaling. A total of 2×10^5 cells from $RIP^{+/+}$ or $RIP^{-/-}$ cell culture were plated on six-well plates and were infected with Ad LacZ or Ad FAK-CD (200 FFU/cell) after 24 h. After 6 and 18 h of treatment, cells were immunostained with anti-FAK 4.47 antibody (A) (white arrowheads mark some focal adhesions), detached cells were counted, and cells were collected for TUNEL assay and Western blot analysis. (B) Detachment and apoptosis data from one of four representative experiments. (C) Western blot (WB) analysis of $RIP^{+/+}$ and $RIP^{-/-}$ lysates treated with Ad LacZ or Ad FAK-CD.

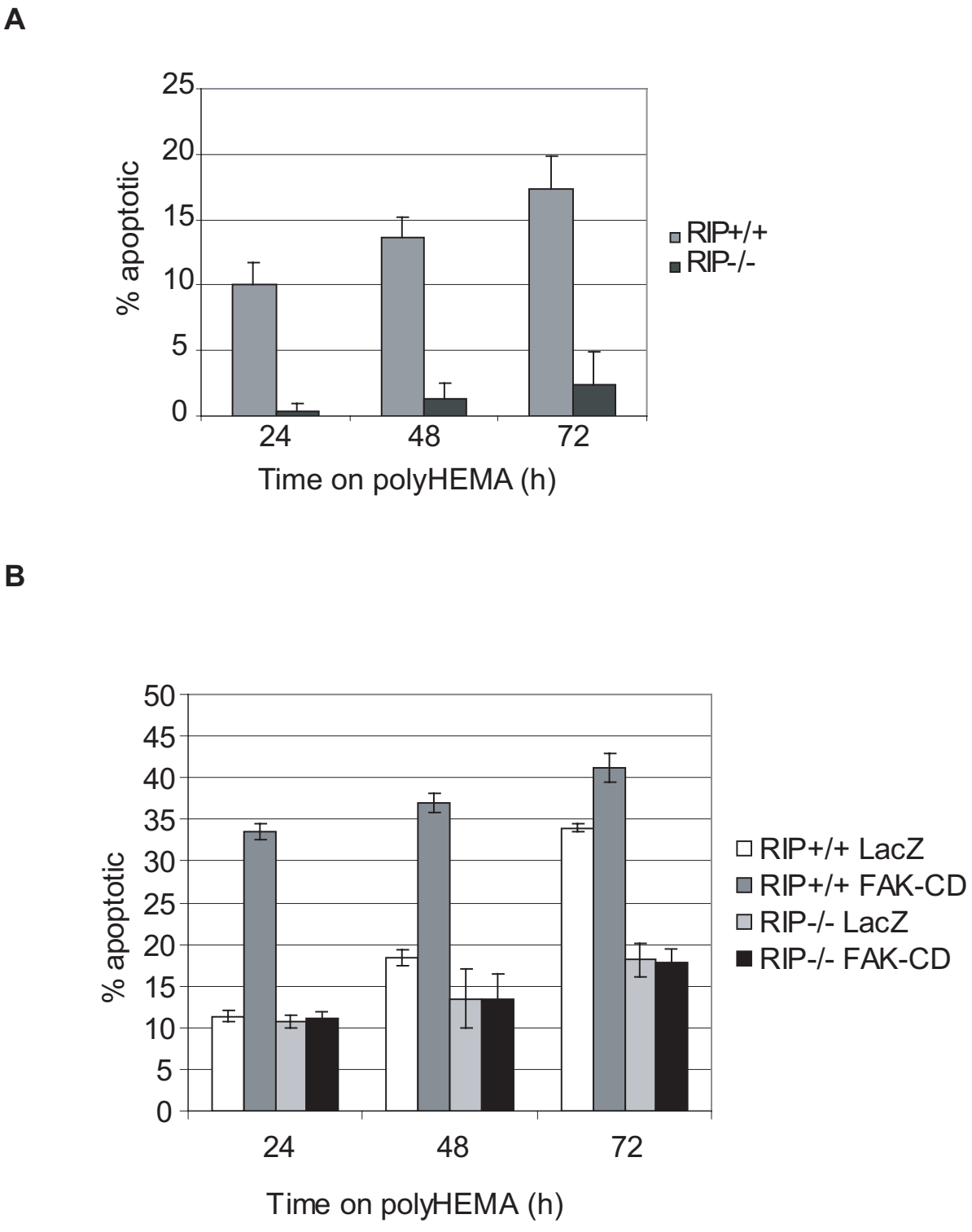

FIG. 5. RIP is involved in transducing the detachment-induced proapoptotic signals. (A) A total of 2×10^6 $RIP^{+/+}$ and $RIP^{-/-}$ cells were plated on tissue culture dishes coated with polyHEMA and were grown in complete medium for 24 to 72 h. TUNEL assay and Hoechst staining were performed at the indicated time points. TUNEL assay data from one of three representative experiments are shown. (B) A total of 2×10^5 $RIP^{+/+}$ and $RIP^{-/-}$ cells were plated on tissue culture dishes coated with polyHEMA and were grown in complete medium for 24 to 72 h and then transduced with either Ad LacZ control or Ad FAK-CD for 24 h. TUNEL assay and Hoechst staining were performed at the indicated time points. TUNEL assay data from one of three representative experiments are shown.

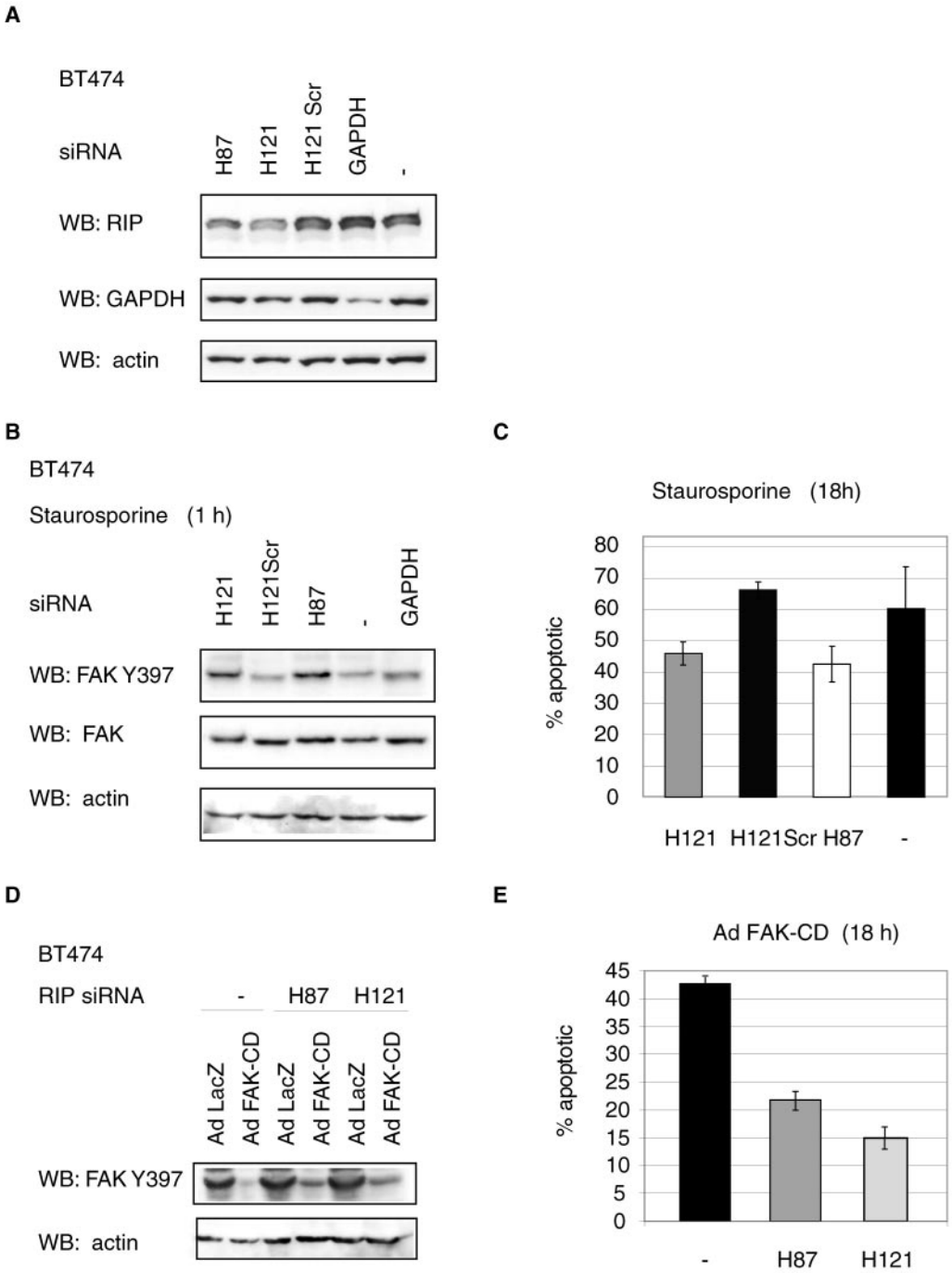

FIG. 6. Downregulation of RIP with siRNA leads to higher resistance to apoptosis caused by the attenuation of FAK. A total of 2×10^5 BT474 cells were plated on six-well plates and were transfected with siRNA (25 nM) after 24 h. (A) Forty-eight hours after transfection, cells were collected and analyzed by Western blotting (WB). Controls: scrambled RIP H121 siRNA (H121Scr), GAPDH siRNA, and cells treated with transfection reagent alone (lanes 3, 4, and 5, respectively). (B and C) After 48 h of transfection, cells were treated with 200 nM staurosporine and collected 1 h later to analyze tyrosine phosphorylation of FAK (B) or collected 18 h later for TUNEL assay (C). (D and E) After 48 h of transfection, adenoviral infection was performed and Ad LacZ and Ad FAK-CD were added to the cell cultures (200 FFU/cell). After 18 h of infection, detached cells were counted, and cells were collected for TUNEL assay and Western blot analysis. (D) Western blot analysis with anti-FAK phosphotyrosine Y397 antibody. (E) TUNEL assay data from one of three representative experiments.

shown that RIP may provide proapoptotic signals in some cases or antiapoptotic signals in others (14, 23, 28). Thus, our model suggests that the upregulation of FAK expression in cancer cells shifts the FAK-RIP balance towards resistance to apoptosis and promotes survival of the cells. Finally, these results may provide the link between FAK and the death receptor for the phenomenon of anoikis. Tumor cells have been postulated to be resistant to anoikis to allow them to survive during the processes of invasion and metastasis that require anchorage-independent survival (15). As tumor cells become detached and proceed with invasion and metastasis, our model predicts that FAK would bind to RIP and provide survival signals to these cells. Thus, this proposed mechanism suggests that targeting the FAK-RIP association might provide a novel approach for cancer therapeutics.

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