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Bit-1 Mediates Integrin-dependent Cell Survival through Activation of the NFκB Pathway*

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Loss of properly regulated cell death and cell survival pathways can contribute to the development of cancer and cancer metastasis. Cell survival signals are modulated by many different receptors, including integrins. Bit-1 is an effector of anoikis (cell death due to loss of attachment) in suspended cells. The anoikis function of Bit-1 can be counteracted by integrin-mediated cell attachment. Here, we explored integrin regulation of Bit-1 in adherent cells. We show that knockdown of endogenous Bit-1 in adherent cells decreased cell survival and re-expression of Bit-1 abrogated this effect. Furthermore, reduction of Bit-1 promoted both staurosporine and serum-deprivation induced apoptosis. Indeed knockdown of Bit-1 in these cells led to increased apoptosis as determined by caspase-3 activation and positive TUNEL staining. Bit-1 expression protected cells from apoptosis by increasing phospho-IκB levels and subsequently *bcl-2* gene transcription. Protection from apoptosis under serum-free conditions correlated with *bcl-2* transcription and Bcl-2 protein expression. Finally, Bit-1-mediated regulation of *bcl-2* was dependent on focal adhesion kinase, PI3K, and AKT. Thus, we have elucidated an integrin-controlled pathway in which Bit-1 is, in part, responsible for the survival effects of cell-ECM interactions.

Adherent cells require attachment to the extracellular matrix (ECM)[2] for survival. Cell survival signals are modulated by many different receptors, including receptor tyrosine kinases and integrins. Integrins are heterodimeric transmembrane receptors that bind to ECM proteins and convey anchorage-dependent signals regulating normal cell survival. Integrins themselves can influence cell survival through more than one pathway (1). Integrins suppress apoptosis in attached cells by activating signaling pathways that promote survival and inactivating the ones that promote apoptosis. Apoptosis that results from a loss of integrin-mediated cell attachment to the ECM is called anoikis (2). The ability to prevent anoikis varies among integrins. Cell attachment by the $\alpha5\beta1$ and $\alpha v\beta3$ integrins protects CHO cells from apoptosis whereas the $\alpha v\beta1$ integrin does not (3). Similarly, $\alpha5\beta1$-mediated attachment protects cultured HT29 colon carcinoma cells from apoptosis caused by serum deprivation (4) and neuronal cells from β-amyloid-induced apoptosis (5). The $\alpha1\beta1$ and $\alpha v\beta3$ integrins can also activate an anti-anoikis pathway (6). A number of integrin-signaling molecules regulate anoikis, including focal adhesion kinase (FAK) and PI3K/AKT (1, 7). In attached cells, the $\alpha5\beta1$ and $\alpha v\beta3$ integrins activate a FAK/PI3K/AKT/BCL-2 survival pathway (3).

Tumor cells are more resistant to anoikis than normal cells. This may provide a survival advantage to malignant cells during invasion and metastasis because these cells may bypass integrin-dependent survival signals (8). Although growth factors can activate some of the same signaling pathways, growth factors cannot substitute for integrin-mediated attachment (9, 10). Therefore integrin-specific signaling pathways are an important component of cell survival and the regulation of apoptosis.

Bit-1 is a protein that appears to be a part of an integrin-specific signaling pathway (11). It is a 179-amino acid protein with a known structure for its putative active site (12). Bit-1 is a mitochondrial protein that is released from the mitochondria upon placing cells in suspension in serum containing media. Moreover, when Bit-1 is artificially targeted to the cytoplasm (by mutation) in attached cells, it binds to AES, a small Groucho/TLE (transducin-like enhancer of split) protein. This complex promotes caspase-independent cell death (11). In this way, Bit-1 acts as a downstream activator of anoikis. Recently, the serine/threonine kinase PKD1 was shown to phosphorylate Bit-1 on two serines (Ser^5 and Ser^{87}) in a mutant form of Bit-1 that is confined to the cytoplasm (13), suggesting that phosphorylation of Bit-1 may affect its function. Overexpression of Bit-1 was also found to impair Erk phosphorylation (14).

Wild type Bit-1 located at the mitochondria does not bind to the AES complex or promote cell death (11). Bit-1 conditional knock-out mice are born smaller than wild type littermates, develop a runting syndrome and die within the first 2 weeks of life (14). Moreover, the KO mouse embryonic fibroblasts are more sensitive to staurosporine-mediated mitochondrial apoptosis compared with wild type mouse embryonic fibroblasts (14), suggesting a survival role for Bit-1 in attached cells.

In attached cells, the signaling pathways through which integrins signal to Bit-1 and the downstream signaling components are completely unknown. In the work reported here, we

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[2] The abbreviations used are: ECM, extracellular matrix; FAK, focal adhesion kinase; FRNK, FAK-related non-kinase.

set out to explore the integrin regulation of Bit-1 in adherent cells. We hypothesized that Bit-1 regulates adhesion-dependent cell survival. Indeed, we found that in attached cells, Bit-1 protects from serum deprivation-mediated apoptosis and staurosporine-induced mitochondrial apoptosis by promoting phosphorylation of IκB and subsequently *bcl-2* gene transcription. Knockdown of endogenous Bit-1 by siRNA promoted apoptosis of cells attached to fibronectin and enhanced staurosporine-induced mitochondrial apoptosis as determined by caspase-3 activation and ApopTag positive staining. In addition, shRNA reduction of Bit-1 promoted apoptosis when the cells were attached to fibronectin but not when cells were attached to collagen IV. Bit-1 mediated up-regulation of NFκB appears to be downstream of FAK and PI3K/AKT. Thus, our data suggest a novel function for Bit-1 as a regulator of integrin-mediated cell survival in cells attached to the ECM.

EXPERIMENTAL PROCEDURES

Cells—The CHO-B2 cells deficient in $\alpha5\beta1$ (CHO-B2) were from Dr. Rudolf Juliano (School of Medicine, University of North Carolina, Chapel Hill, NC) (15). The CHO-B2/$\alpha5\beta1$, CHO-B2/$\alpha5\Delta c\beta1$, and the CHO-B2/$\alpha v\beta1$ were described previously (3). Briefly, CHO-B2/$\alpha5\beta1$ and CHO-B2/$\alpha v\beta1$ cells were generated by introducing cDNAs coding for the $\alpha5$ and αv integrin subunits into $\alpha5\beta1$-deficient CHO-B2 cells (15, 16). The CHO-B2/$\alpha5\Delta c\beta1$ cells were obtained by transfection of the B2-CHO cells with a truncated $\alpha5$ construct that lacked the cytoplasmic domain. Transfectant expressing the integrin were cloned and expanded (17). All cells were maintained in a modified Eagles' medium (Invitrogen) supplemented with 10% fetal bovine serum and glutamine/penicillin-streptomycin (Sigma). G418 was added to the medium of transfected cells at a concentration of 250 μg/ml. HeLa cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum and glutamine/penicillin-streptomycin (Sigma).

Reagents—The cDNAs for constitutively active PI3K (P110-CAAX) and dominant negative PI3K (p85D) cDNAs were from Dr. Kristiina Vuori (The Burnham Institute, La Jolla, CA). CD2-FAK and FAK-related non-kinase (FRNK) cDNAs were from Dr. Jun-Lin Guan (Cornell University College of Veterinary Medicine, Ithaca, NY) (18).

Plasmid Constructs—The *bcl-2* promoter was from Dr. John C. Reed (The Burnham Institute, La Jolla, CA) (17). The *bcl-2* promoter-GFP construct was described previously (3). Briefly, the full-length human *bcl-2* promoter was cloned into the BglII and HindIII cloning sites of the promoter-less enhanced green fluorescent protein vector (pEGFP-1; Clontech). The construct was sequenced to determine that the *bcl-2* promoter-GFP construct was in frame. The pcDNA3 vector was used as a control.

Transient Transfection—Cells were plated on 75-mm^2 bacterial culture plates that had been coated with human fibronectin (25 μg/ml; Invitrogen) overnight at 4 °C. Cells were plated at 1×10^6 cells/plate and allowed to attach at 37 °C for 4 h. Spread cells were rinsed with PBS and transiently transfected with various cDNAs using the Lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen). Transfection efficiency was measured using immunoblotting as described below.

Analysis of bcl-2 Promoter-enhanced GFP Construct—The *bcl-2* promoter up-regulation was analyzed in transiently transfected cells at 24 and 48 h. Cells were examined under a Zeiss fluorescence microscope at various time points to detect the GFP marker. A FluoroMax-2 fluorimeter (ISA, Jobin Yvon-Spex, Horiba) was used to quantitate GFP fluorescence within cells. At various time points, cells were trypsinized (trypsin-EDTA; MediaTech), washed twice with serum-containing medium and washed once with PBS. Cells were resuspended in 2 ml of PBS, and GFP fluorescence was measured at 467 nm excitation and 507 nm emission.

Cell Viability Assay—Cell viability was assessed using the MTT assay (19). Cells cultured in microtiter wells were pulsed with 25 μl of a 2.5 mg/ml MTT stock (Sigma) in PBS and incubated for 4 h, after which 100 μl of a solution containing 10% SDS, 0.01 N HCl was added. The plates were then incubated overnight, and absorption was read on a VICTOR 3 multi-label plate reader (PerkinElmer Life Sciences). Using a reference wavelength of 650 nm and a test wavelength of 590 nm. Test reagents were added to medium alone to provide a blank.

Staurosporine-mediated Apoptosis—Staurosporine (2 μM; Sigma) was added to adherent cells in serum-containing media. 24 h later, the cells were analyzed by either the MTT assay (described above), immunoblotting for caspase-3 activation, or Apoptag assay (described below).

RNA Interference of Bit-1—A Dharmacon si-genome SMARTpool (Thermo Fisher Scientific) consisting of four Bit-1-specific siRNAs and control siRNAs was used to knock down Bit-1 expression levels. For transient transfection experiments, 2×10^5 HeLa cells were transfected with 25 μM of Bit-1 SMARTpool using the Lipofectamine 2000 transfection reagent (Invitrogen). Three days post-transfection, cells were collected for immunoblotting or for survival assays. To overexpress Bit-1 after siRNA transfection, the control or Bit-1 siRNA treated cells were transfected with 3 μg of the empty vector or the WT-Bit-1 construct 3 days post-siRNA transfection. 24 h later, cells were collected. For stable Bit-1 knockdown, a GIPZ lentiviral shRNAmir (Open Biosystems) was developed in HeLa cells and in H9c2 cells as described in the manufacturer's protocol. Briefly, HeLa or H9c2 cells were transduced with lentiviral particles containing shRNAmir to Bit-1 or empty vector and non-silencing shRNAmir was transduced as a control and subsequently selected with puromycin.

Immunoblotting—HeLa, H9c2, human coronary arterial endothelial cells (HCAECs), CHO-B2/$\alpha5\beta1$, CHO-B2/$\alpha5\Delta c\beta1$, and CHO-B2/$\alpha v\beta1$ cells were lysed with lysis buffer containing 50 mM Tris-HCL, 3 mM EDTA, 0.5% Triton X-100, pH 7.0, 0.5 mM dithiothreitol, and protease inhibitors (Complete Protease Inhibitor Tablets, Roche Applied Science). Cell lysates were cleared by centrifugation at 28,000 rpm for 10 min at 4 °C. Equal amounts of cell lysates were resolved on 4–12% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes and immunoblotted. Membranes were blocked for 1 h with blocking buffer (3% BSA in TBS-T) and incubated with either a rabbit polyclonal anti-Bit-1 antibody (developed at Washington Biotechnology (Columbia, MD) using the Bit-1 amino acid sequence GPADLIDKVTAGHLKL), anti-Bcl-2 antibody (Santa Cruz

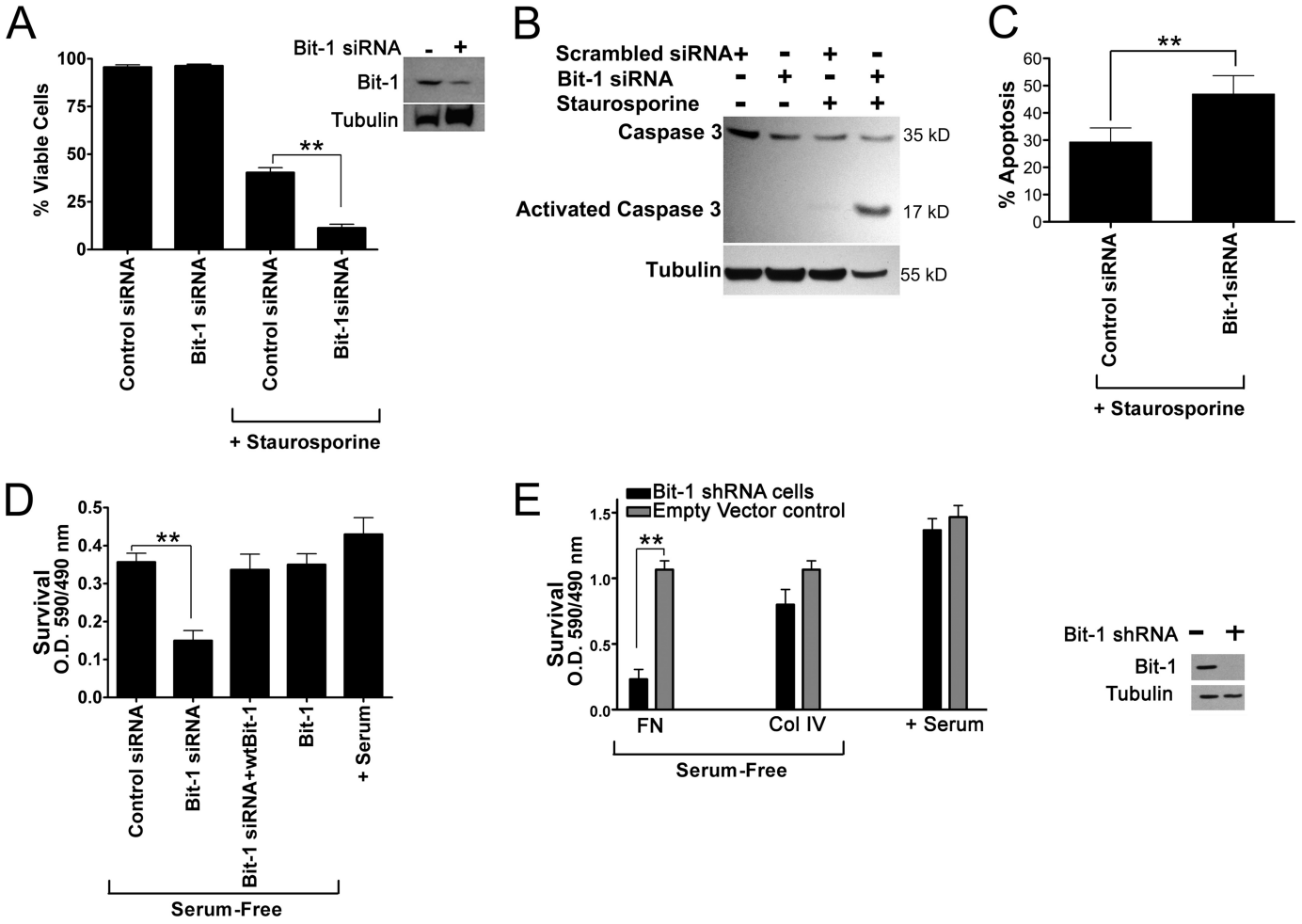

FIGURE 1. **Bit-1 knockdown by siRNA or shRNA enhances apoptosis in a tumor cell line.** *A*, HeLa cells transiently transfected with Bit-1 siRNA or a scrambled siRNA control were plated on fibronectin (*FN*) in serum-containing media and allowed to attach prior to adding staurosporine. Cell viability ± staurosporine was measured 24 h later using the MTT assay. Values represent the mean ± S.D. ($n = 9$). Statistically significant differences are reported in the graph as p values (Student's t test). **, $p < 0.01$. *Inset*, Western blot of Bit-1 expression ± Bit-1 siRNA. *B*, HeLa cells transfected with Bit-1 siRNA or scrambled siRNA were plated on fibronectin and treated with staurosporine. Cells were then lysed, and cell lysates were immunoblotted for caspase-3 activation using an anti-caspase-3 antibody that detects uncleaved and active caspase-3 (17 kDa). Tubulin was used as loading control. Data are representative of at least three separate experiments. *C*, HeLa cells transfected with Bit-1 siRNA or scrambled siRNA plated on fibronectin were treated with staurosporine. The cells were then analyzed using the ApopTag *in situ* apoptosis detection kit. Positively stained cells were detected by FACS analysis. Values represent the mean ± S.D. ($n = 9$). Statistically significant differences are reported in the graph as p values (Student's t test). **, $p < 0.01$. *D*, HeLa cells transfected with Bit-1 siRNA, scrambled siRNA, or Bit-1 siRNA + wild type Bit-1 (WT Bit-1) were plated on fibronectin in serum-free conditions and examined for cell viability under serum-free conditions using the MTT assay. Values represent the mean ± S.D. ($n = 9$). Statistically significant differences are reported in the graph as p values (Student's t test). **, $p < 0.01$. *E*, exponentially growing, stable Bit-1 knockdown cells (Bit-1 shRNA cells) and empty vector control cells were harvested and subjected to immunoblotting (*inset*) and plated on fibronectin or collagen IV in serum-free conditions and analyzed 72 h later for cell survival using the MTT assay. Values represent the mean ± S.D. ($n = 9$). Statistically significant differences are reported in the graph as p values (Student's t test). **, $p < 0.01$. *O.D.*, optical density.

Biotechnology, Santa Cruz, CA), anti-caspase-3 (Cell Signaling, Beverly, MA), an anti-HA antibody (Santa Cruz Biotechnology), anti-AKT and anti-phospho-AKT (Cell Signaling), or anti-IκB and anti-phospho-IκB (Cell Signaling) for 2 h. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) for 1 h. Immunoblots were developed by ECL (GE Healthcare).

Immunostaining—H9c2 cells were plated on fibronectin (15 μg/ml) or collagen IV (15 μg/ml) coated slides in serum-free media. At various time points, from 15 min to 24 h later, cells were subsequently fixed in 4% paraformaldehyde for 15 min, washed 1× in PBS, permeabilized (0.2% Triton X-100, 5 min at 37 °C), and blocked (PBS + 3% BSA for 30 min) prior to incubation with a rabbit anti-Bit-1 polyclonal antibody (1:100; ProSci) or anti-FAK antibody (1:100; ProSci) for 1 h at 37 °C. After three PBS washes, cells were incubated with Alexa Fluor anti-rabbit 488 nm (green) or anti-mouse 594 nm (red) secondary antibodies (1:500, 1 h; 37 °C; Molecular Probes). After three final washes in PBS, slides were counterstained with DAPI, coverslipped, and imaged using a Zeiss Axiovert 200m inverted fluorescent microscope (5). For Mitotracker Red analysis, cells were treated as described below and then immunostained for Bit-1 and counterstained with Draq5 (Biostatus Limited) to detect nuclei.

Immunoprecipitation—H9c2 and HCAEC cells were plated on 100-mm plates coated with fibronectin or collagen IV, and at various time points, cells were lysed in lysis buffer containing 50 mM Tris-HCl, 50 mM NaCl, 0.5% Triton X-100, 10% glycerol, 0.1% BSA, and protease inhibitors (0.1 unit/ml aprotinin, 10 μg of leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride).

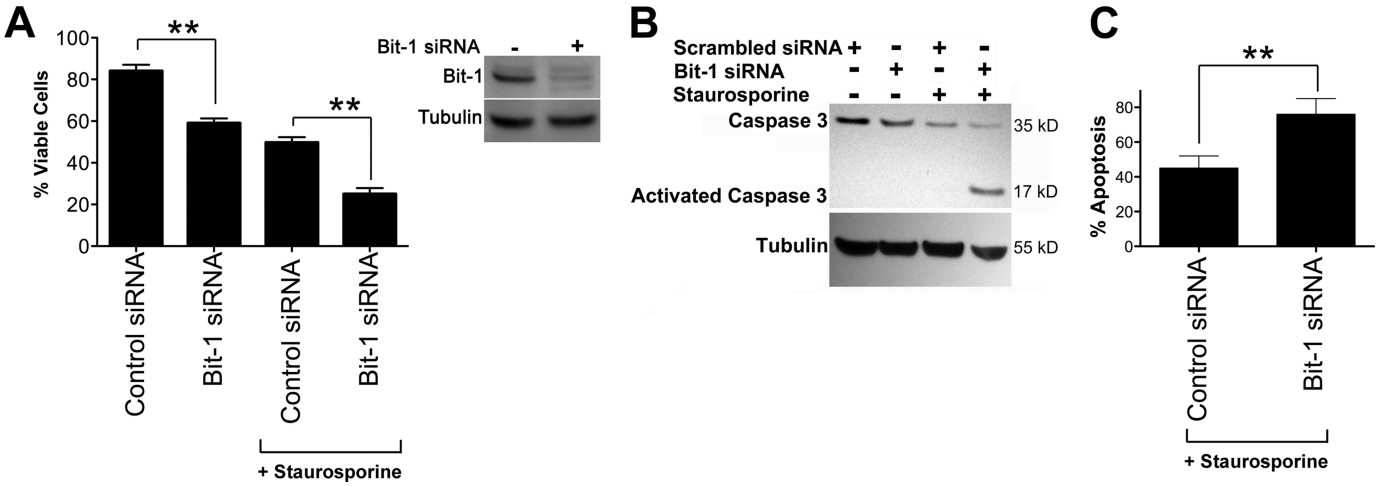

FIGURE 2. **Bit-1 knockdown by siRNA enhances staurosporine-mediated apoptosis in normal endothelial cells.** *A*, HCAECs transiently transfected with Bit-1 siRNA or a scrambled siRNA control were plated on fibronectin in serum-containing media and allowed to attach prior to adding staurosporine. Cell viability ± staurosporine was measured 24 h later using the MTT assay. Values represent the mean ± S.D. ($n = 9$). Statistically significant differences are reported in the graph as p values (Student's t test). **, $p < 0.01$. *Inset*, Western blot of Bit-1 expression ± Bit-1 siRNA. *B*, HCAECs transfected with Bit-1 siRNA or scrambled siRNA were plated on fibronectin and treated with staurosporine prior to immunoblotting cell lysates for caspase-3 activation using an anti-caspase-3 antibody that detects the uncleaved and active caspase-3 (17 kDa). Tubulin was used as loading control. Data are representative of at least three separate experiments. *C*, HCAECs transfected with Bit-1 siRNA or scrambled siRNA plated on fibronectin were treated with staurosporine. The cells were then analyzed using the ApopTag *in situ* apoptosis detection kit. Positively stained cells were detected by FACS analysis. Values represent the mean ± S.D. ($n = 9$). Statistically significant differences are reported in the graph as p values (Student's t test). **, $p < 0.01$.

Lysates were centrifuged at 14,000 × rpm for 10 min. Antibodies at a concentration of 2–4 μg were added to lysates containing an equal amount of proteins and incubated overnight at 4 °C. To precipitate the antibody-antigen complex, Protein A/G PLUS agarose beads (Santa Cruz Biotechnology) were added to lysates and incubated for 4 h at 4 °C. Samples treated with IgG beads in the absence of antibody were used as a negative control. The immunoprecipitates were pelleted by centrifugation and washed twice with PBS. Beads were boiled in sample buffer and separated on SDS-PAGE gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane and subject to immunoblotting as described below.

ApopTag Staining—HCAEC and HeLa cells were allowed to attach to fibronectin in serum-containing media prior to the addition of staurosporine (as described above). Cells were then fixed in 1% paraformaldehyde in PBS, permeabilized in 70% ethanol, and stained with the ApopTag reagent (apoptosis *in situ* detection kit, Millipore, Temecula, CA) and propidium iodide per the kit protocol. The stained cells were analyzed by flow cytometry.

Mitotracker Red—H9C2 cells were allowed to attach to fibronectin or collagen IV in serum-free conditions for 1 h prior to being treated with serum-free media containing 200 nM Mitotracker Red CMXROS (Invitrogen) for 20 min. After treatment, cells were washed once in PBS and subsequently fixed in 4% paraformaldehyde for 15 min. Cells were immunostained for Bit-1 as described above.

RESULTS

Bit-1 Is Required for Cell Survival in Tumor Cells—Wild type Bit-1 located at the mitochondria does not bind to the cytoplasmic AES complex or promote cell death (11). Bit-1 conditional knock-out mice are born smaller than their wild type littermates, develop a runting syndrome, and die within the first 2 weeks of life (14), suggesting a survival role for wild type Bit-1 in attached cells. Moreover, Bit-1 was isolated by expression cloning as a protein that can up-regulate Bcl-2 expression in integrin-compromised cells (11). If Bit-1 is involved actively in promoting cell survival, then elimination of Bit-1 in adherent cells should increase cell death. To test this hypothesis, we examined whether knockdown of Bit-1 altered the sensitivity of cells to staurosporine stress. To determine whether Bit-1 expression correlated to cell survival, we assessed cell survival by the MTT assay, which measures cell viability by detecting the ability of a mitochondrial enzyme to reduce its substrate. We chose to examine survival in the HeLa cervical tumor cell line because increased cell survival can contribute to cancer transformation and metastasis. Indeed, we found that HeLa cells expressing endogenous Bit-1 were more resistant to staurosporine-induced apoptosis (Fig. 1*A*) compared with cells that had endogenous Bit-1 levels knocked down by siRNA (Fig. 1*A*). To investigate whether this survival was a result of Bit-1 protecting cells from apoptosis, we assayed for caspase-3 activity. Upon treatment with an apoptotic stimulus inactive caspase-3 is cleaved into an active 17-kDa peptide (20). Therefore, we examined whether knockdown of Bit-1 enhanced caspase-3 activation upon staurosporine treatment. We found that staurosporine-treated cells in which Bit-1 levels were reduced had increased levels of active caspase-3 (Fig. 1*B*). To confirm the role of Bit-1 in regulating apoptosis, we further assayed for increased ApopTag reactivity using flow cytometry. ApopTag is a fluorescein-conjugated antibody that binds to digoxigenin-tagged 3-OH DNA ends generated by DNA fragmentation (21). Cells expressing reduced Bit-1 had increased DNA fragmentation due to apoptosis upon treatment with staurosporine (Fig. 1*C*). Taken together, these findings suggest that in cells attached to fibronectin Bit-1 is involved in survival signaling.

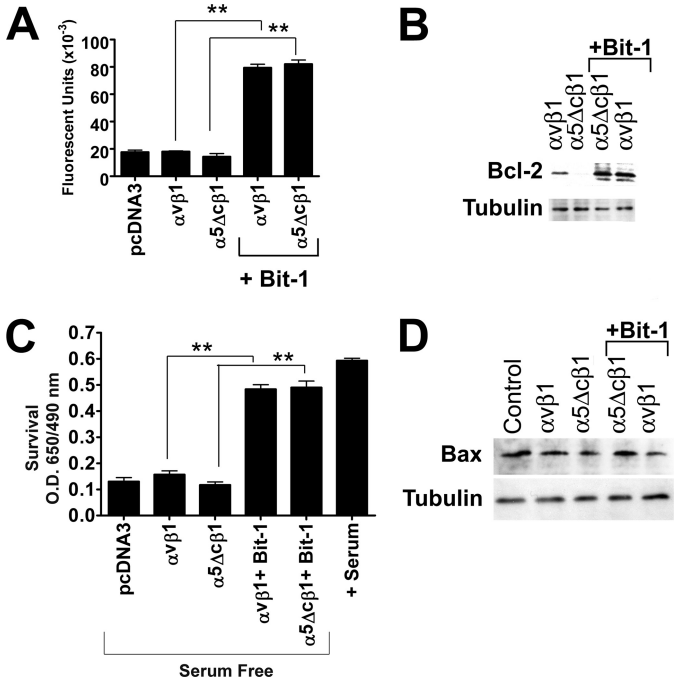

FIGURE 3. **Bit-1 up-regulates *bcl-2* at both the transcription and protein levels.** CHO-B2 cells engineered to express various integrins were transfected with *bcl-2* promoter-GFP construct and plated on fibronectin in serum-free conditions. The bcl-2 promoter drives GFP expression. *A*, quantitation of GFP fluorescence 48 h after transfection. *B*, Western blot of Bcl-2 protein expression ± Bit-1. *C*, effect of Bit-1 on cell viability. Cultures were examined for viability by the reduction of MTT after 72 h. For *A* and *C*, values represent the mean ± S.D. ($n = 9$). Statistically significant differences are reported in the graph as *p* values (Student's *t* test). **, $p < 0.01$. *D*, Western blot of Bax protein expression. Tubulin was used as loading control. *B* and *D* are representative of three independent experiments. *O.D.*, optical density.

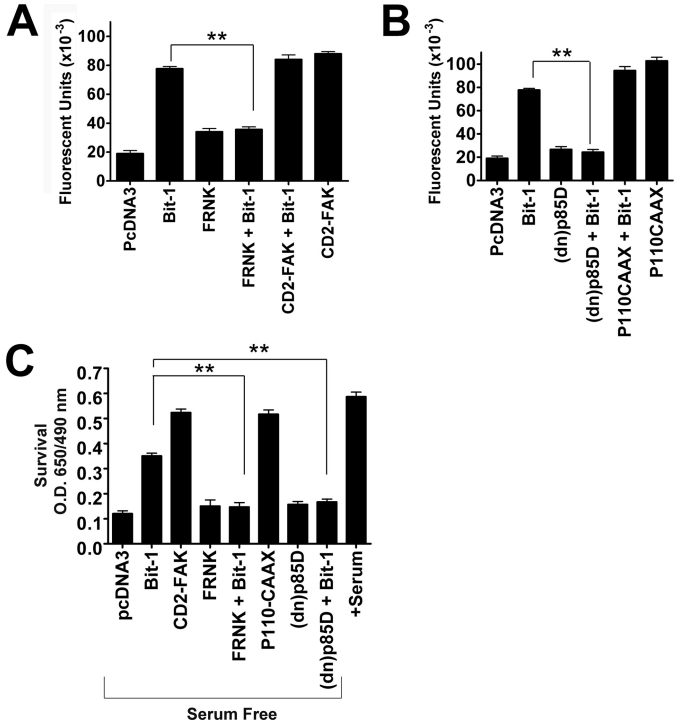

FIGURE 4. **A FAK/PI3K pathway mediates Bit-1 activation of *bcl-2* transcription and survival in attached cells.** *A*, *bcl-2* promoter/GFP fluorescence is enhanced in CHO/$\alpha5\Delta c\beta1$ cells transfected with the *bcl-2* promoter-GFP, active FAK (CD2-FAK) or WT Bit-1 plated on fibronectin in serum-free media. Up-regulation of *bcl-2*/GFP fluorescence by Bit-1 in $\alpha5\Delta c\beta1$ is suppressed by FRNK that acts as a dominant negative FAK. FRNK or the control pcDNA3 vector does not up-regulate *bcl-2*/GFP fluorescence. Constitutively active FAK (CD2-FAK) up-regulates *bcl-2*/GFP fluorescence. *B*, Bit-1 up-regulates *bcl-2* transcription and dominant negative PI3K (p85D) suppresses it. *bcl-2*/GFP fluorescence is up-regulated by Bit-1. Dominant negative PI3K (p85D) suppresses GFP fluorescence induced by Bit-1 in the CHO/$\alpha5\Delta c\beta1$ cells. Constitutively active PI3K (P110-CAAX) up-regulates *bcl-2*/GFP fluorescence. *bcl-2* promoter-GFP fluorescence was measured 48 h post-transfection. *C*, CHO/$\alpha5\Delta c\beta1$ cultures transiently transfected with the indicated constructs were plated on fibronectin. After 72 h, cultures were examined for cell viability under serum-free conditions using the MTT assay. Dominant negative FAK (*dn*; FRNK) and dominant negative PI3K (p85D) suppressed Bit-1 ability to promote cell survival. For *A–C*, values represent the mean ± S.D. ($n = 9$). Statistically significant differences are reported in the graph as *p* values (Student's *t* test). **, $p < 0.01$. *O.D.*, optical density.

siRNA Knockdown of Endogenous Bit-1 Promotes Apoptosis of Cells Attached to Fibronectin—We next examined whether siRNA knockdown of endogenous Bit-1 would affect survival in cells attached to fibronectin via the $\alpha5\beta1$ integrin that elevates *bcl-2* transcription and promotes cell survival under serum-free conditions (3). Endogenous Bit-1 protein levels were knocked down using a Dharmacon SMARTpool consisting of four siRNAs for Bit-1 (Fig. 1*A*, *inset*). Cells in which Bit-1 protein levels were reduced were plated on fibronectin in serum-free conditions. To determine whether Bit-1 expression corresponded to cell survival, we assessed cell survival by the MTT assay. Cells with reduced Bit-1 were less viable when cultured on fibronectin in serum-free conditions (Fig. 1*D*), whereas control cells receiving scrambled siRNA survived (Fig. 1*D*). Moreover, re-expressing Bit-1 rescued the cells under serum-free conditions (Fig. 1*D*).

To further evaluate the effect of down-regulating Bit-1 on cell survival in attached cells, we developed a stable Bit-1 knockdown clone of HeLa cells (Fig. 1*E*). We used these cells to examine whether the Bit-1 cell survival function was integrin-specific. The Bit-1 shRNA knockdown cells attached to fibronectin exhibited increased sensitivity to serum deprivation-mediated apoptosis compared with both the control cells and to the knockdown cells attached to collagen IV (Fig. 1*E*). Taken together, these data suggest that Bit-1 is involved in survival signals that are downstream of a fibronectin-binding integrin (*e.g.* $\alpha5\beta1$) in attached cells.

Bit-1 Protects Normal Endothelial Cells from Apoptosis—Elimination of Bit-1 in adherent HeLa cells increased cell death. Because tumor cells are more resistant to apoptosis compared with normal cells, we next wanted to determine whether there is a role for Bit-1 in cell survival in normal cells. Endothelial cells are particularly sensitive to anoikis. Therefore, we examined whether knockdown of Bit-1 altered the sensitivity of endothelial cells to staurosporine. To determine whether Bit-1 expression correlated to cell survival, we assessed cell survival by the MTT assay. Indeed, we found that endothelial cells expressing endogenous Bit-1 were more resistant to staurosporine-induced apoptosis compared with cells treated with Bit-1 siRNA (Fig. 2*A*). To investigate whether this was due specifically to Bit-1 effects on apoptosis, we examined caspase-3 activity in these cells. We found that siRNA knockdown of Bit-1 led to increased levels of active caspase-3 in staurosporine-treated endothelial cells compared with control cells (Fig. 2*B*). To further confirm the role of Bit-1 in regulating apoptosis in these cells, we assayed for ApopTag reactivity by flow cytometry.

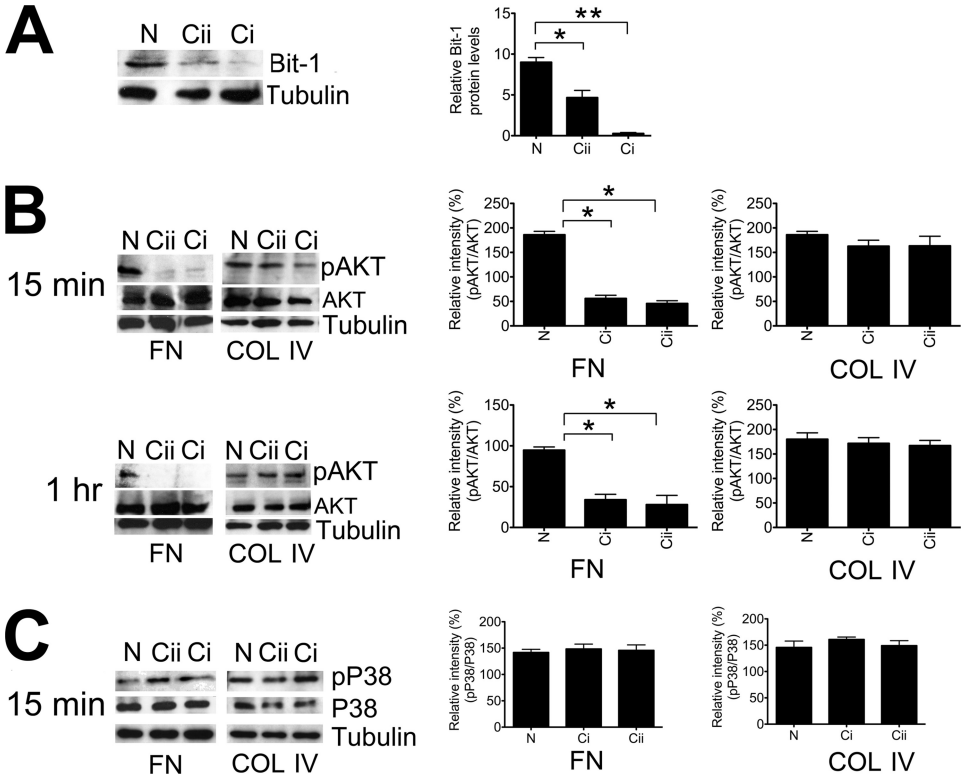

FIGURE 5. **Knockdown of Bit-1 decreases pAKT levels in cells attached to fibronectin but not when attached to collagen IV.** Exponentially growing, stable Bit-1 shRNA knockdown H9c2 cells (*Ci* and *Cii* are shRNA stable clones) and empty vector control (*N*) were harvested and subjected to **(***A*; *left***)** immunoblotting for Bit-1 levels using a polyclonal anti-Bit-1 antibody. *Right*, relative Bit-1 protein levels. Three independent replicates of the Western blot analysis were subjected to quantification by densitometry and normalized to the tubulin signal that was used as loading control. The results were expressed as relative protein levels as mean ± S.D. Statistically significant differences are reported in the graph as *p* values (Student's *t* test). *, $p < 0.05$; **, $p < 0.01$. *B*, cells were plated on fibronectin or collagen IV in serum-free conditions for either 15 min or 1 h. At the time points analyzed, cells were lysed and subjected to immunoblotting for phospho-AKT (pAKT) and total AKT (AKT). Tubulin was used as the loading control. *Right*, the relative pAKT/AKT intensity was determined. *, $p < 0.01$ (Student's *t* test). The values represent the average of three independent experiments. *C*, loss of Bit-1 did not alter phospho-p38 (*pP38*) or total p38 (*tP38*) levels in attached cells. The values represent the average of three independent experiments. *COL IV*, collagen IV; *FN*, fibronectin.

Knockdown of Bit-1 levels demonstrated increased apoptotic DNA fragmentation upon treatment with staurosporine (Fig. 2*C*). Taken together, these findings support a role for Bit-1 in survival signaling in normal adherent cells.

Bit-1 Protects Cells from Apoptosis by Up-regulating bcl-2 Transcription and Expression—The $\alpha5\beta1$ integrin can support cell survival in part by up-regulating Bcl-2 transcription downstream of FAK activation (3). To determine whether Bit-1 is involved in this integrin survival pathway, we made use of the previously examined CHO cell lines, which express full-length human integrin $\alpha5$ (CHO-B2/$\alpha5\beta1$), the $\alpha5$ subunit lacking the cytoplasmic domain (CHO-B2/$\alpha5\Delta c\beta1$), or the αv subunit (CHO-B2/$\alpha v\beta1$(15, 16). These cell lines were transiently transfected with a reporter construct in which the *bcl-2* promoter drives expression of GFP (3). Thus, activation of *bcl-2* transcription downstream of integrins can be measured by GFP fluorescence. We used this bcl-2 promoter-GFP marker to examine whether Bit-1 expression up-regulates bcl-2 transcription. These cells are thus particularly useful for determining whether a protein or pathway can substitute for the lost integrin survival signal. CHO/$\alpha v\beta1$ and CHO/$\alpha5\Delta c\beta1$ cells transfected with the bcl-2 promoter-GFP reporter construct were plated on fibronectin in serum-free conditions. Activation of *bcl-2* transcription was then measured by GFP fluorescence. In agreement with previous results (3), adhesion to fibronectin did not activate *bcl-2* transcription in the CHO/$\alpha v\beta1$ or CHO/$\alpha5\Delta c\beta1$ cells (Fig. 3*A*), whereas expression of Bit-1 in these cells rescued elevated *bcl-2* transcription (Fig. 3*A*) that correlated with increased Bcl-2 protein expression (Fig. 3*B*). The average transfection efficiency was ~70%. The proapoptotic Bcl-2 family member Bax was expressed at similar levels in each of the cell lines, and Bit-1 expression did not change Bax protein levels (Fig. 3*D*)**,** suggesting that Bit-1 acts on Bcl-2 but not on other Bcl-2 family members. These findings suggest that Bit-1 can substitute for downstream integrin survival signaling.

To determine whether Bit-1-mediated increases in Bcl-2 led to increased cell survival, we assessed cell survival by the MTT assay. In agreement with earlier results, CHO/$\alpha v\beta1$ and CHO/$\alpha5\Delta c\beta1$ were not viable when cultured on fibronectin in serum-free conditions (3), whereas expression of Bit-1 in these cells promoted survival for an extended period of time (Fig. 3*C*).

Bit-1 Regulates bcl-2 via a FAK-dependent Pathway—Integrin adhesion to the ECM leads to the rapid recruitment and activation of FAK, and activated FAK can suppress apoptosis in a number of cell types (22). Because FAK mediates integrin-activated *bcl-2* transcription (3) and we show here that Bit-1 up-regulates *bcl-2* transcription, we next examined whether

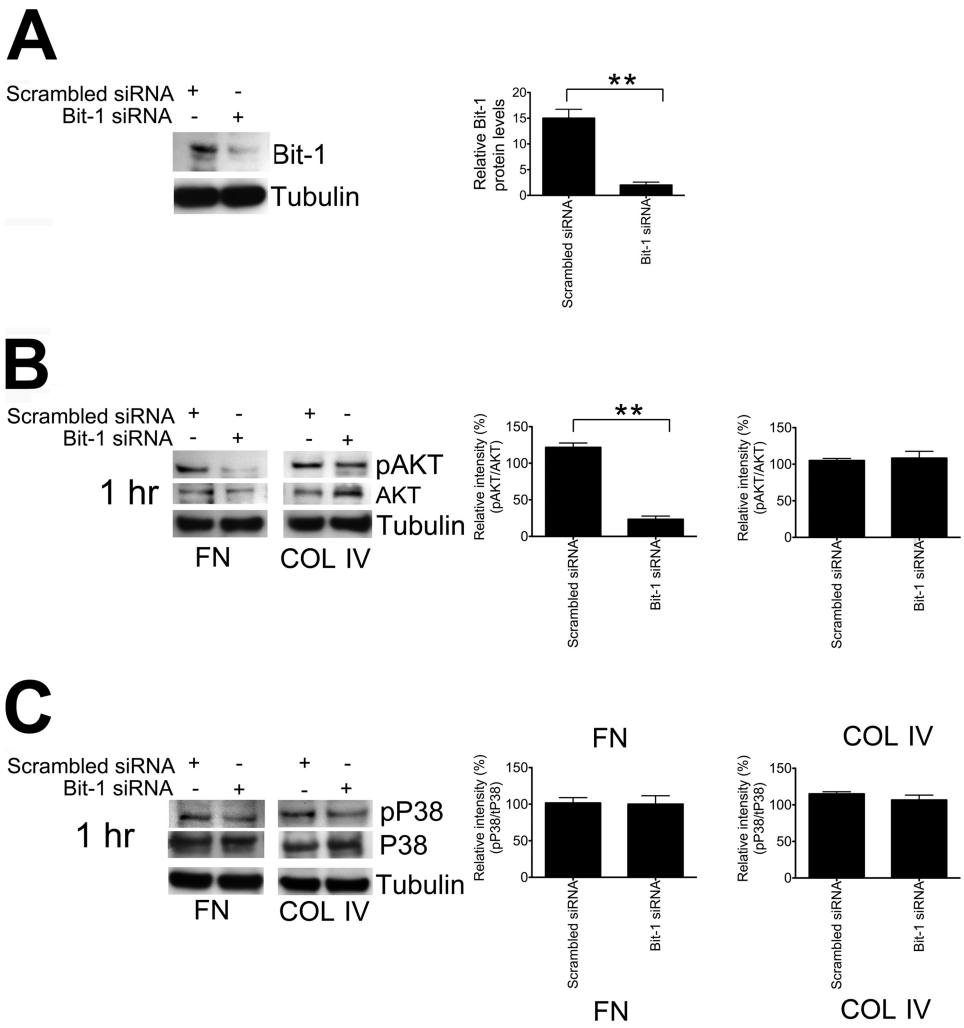

FIGURE 6. **Knockdown of Bit-1 decreases pAKT levels in HeLa cells attached to fibronectin but not when attached to collagen IV.** *A*, HeLa cells transiently transfected with Bit-1 siRNA or a scrambled siRNA control were examined by Western blot for Bit-1 knockdown. Tubulin was used as a loading control. *B*, HeLa cells transiently transfected with Bit-1 siRNA or a scrambled siRNA control were plated on fibronectin (*FN*) or collagen IV (*COL IV*) in serum-free conditions for 1 h. Cells were then lysed and subjected to immunoblotting for phospho-AKT (pAKT) and total AKT (AKT). Tubulin was used as the loading control. *Right*, the relative pAKT/AKT intensity was determined. *, $p < 0.01$ (Student's *t* test). The values represent the average of three independent experiments. *C*, loss of Bit-1 did not alter phospho-p38 (*pP38*) or total p38 (*tP38*) levels in attached HeLa cells. The values represent the average of three independent experiments.

FAK played a role in Bit-1-mediated *bcl-2* up-regulation. In agreement with earlier results, expression of an activated form of FAK (CD2-FAK) caused elevated *bcl-2* transcription in the CHO/α5ΔCβ1 cells (Fig. 4*A*). In contrast, *bcl-2* was not activated in cells expressing FRNK (23), which contains the carboxyl-terminal domain of FAK (Fig. 4*A*). Moreover, FRNK, which acts as a dominant negative form of FAK by interfering with integrin-mediated activation of FAK, blocked the activation of *bcl-2* by Bit-1 (Fig. 4*A*). These results indicate that in attached cells, Bit-1 enhances *bcl-2* expression through an integrin pathway that is dependent on FAK.

Bcl-2 Up-regulation by Bit-1 Requires PI3K—We next examined PI3K involvement. CHO/α5Δcβ1 cells (Fig. 4*B*) expressing activated PI3K (p110-CAAX) exhibited elevated *bcl-2* transcription levels (3). We also tested p85D, the regulatory subunit of PI3K that lacks the binding site for the catalytic subunit p110 and acts as a dominant negative by binding P13K regulatory proteins (24). Forced expression of dominant negative p85D blocked *bcl-2* transcription induced by Bit-1 (Fig. 4*B*). The p85D dominant negative alone had no effect on *bcl-2* transcription (Fig. 4*B*). These results suggest that in attached cells Bit-1 mediated up-regulation of *bcl-2* is dependent on PI3K.

Bit-1-dependent Activation of bcl-2 Transcription Correlates with Cell Survival—We next tested whether expression of Bit-1, activated FAK, or PI3K would prevent apoptosis induced by serum deprivation in the CHO/α5Δcβ1 cells attached to fibronectin. In attached cells, these proteins counteracted the apoptosis-inducing effect of serum withdrawal (Fig. 4*C*). Importantly, cells transfected with Bit-1 in conjunction with FRNK or dominant negative p85D survived poorly without serum (Fig. 4*C*). Thus, in attached cells, Bit-1 promotes cell survival via a FAK-PI3K pathway that up-regulates *bcl-2* expression.

shRNA Knockdown of Bit-1 Decreases Phospho-AKT Levels in Cells Attached to Fibronectin but Not in Cells Attached to Collagen IV—To confirm that Bit-1 is required for integrin-dependent survival signals, we examined whether knockdown of Bit-1

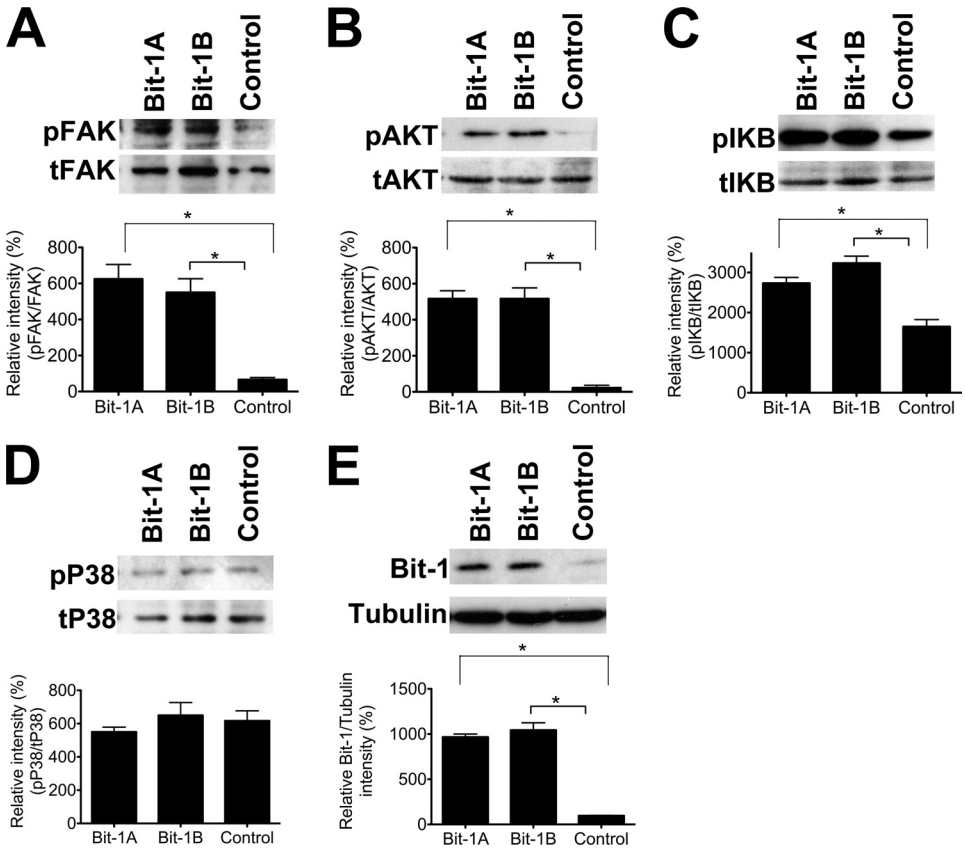

FIGURE 7. **Bit-1 increases pAKT and pIκB levels in HeLa cells.** Two Bit-1-overexpressing HeLa cell lines (*Bit-1A* and *Bit-1B*) or vector control cells plated on fibronectin in serum-free media were lysed and immunoblotted for phospho-FAK (pFAK) and total FAK (*tFAK*) (*A*), phospho-AKT (pAKT) and total AKT (*tAKT*) (*B*), phospho-IκB (pIκB) and total IκB (*tIκB*) (*C*), phospho-p38 and total p38 (*D*) or Bit-1 protein levels (*E*). Phospho-p38 (pP38) and total p38 (*tP38*) levels did not change upon Bit-1 overexpression. Relative intensity of pFAK/total FAK, pAKT/total AKT, pIκB/total IκB were determined. Relative intensity of Bit-1/tubulin and pP38/total P38 was measured. *, $p = <0.05$. (Student's *t* test). Tubulin was used as a loading control. The values represent the average of three independent experiments.

would impact PI3K/AKT signaling. Because *Bit-1* null mice have significant heart defects,[3] we created stable Bit-1 shRNA knockdown H9c2 myoblast clones (Ci, Cii) and non-silencing vector control cells (Fig. 5*A*; *N*). In Bit-1 knockdown clones attached to fibronectin, the phospho-AKT level was decreased significantly compared with the non-silencing control or to the clones attached to collagen IV (Fig. 5B). Phospho-p38 levels were similar in the Bit-1 knockdown cells and controls, suggesting that Bit-1 does not impact the p38 signal transduction pathway (Fig. 5*C*). Taken together, these data suggest that knockdown of Bit-1 blocks AKT activation and subsequent downstream signals when cells are attached to fibronectin but not in cells attached to collagen IV.

As we have previously shown that Bit-1 supports adhesion-dependent survival of HeLa cells, we next examined whether knockdown of Bit-1 in these cells similarly impacts PI3K/AKT signaling. Endogenous Bit-1 protein levels were knocked down using a Dharmacon SMARTpool consisting of four siRNAs for Bit-1 (Fig. 6*A*). Cells in which Bit-1 protein levels were reduced were plated on fibronectin or collagen IV in serum-free conditions. In cells on fibronectin treated with Bit-1 siRNA, the phospho-AKT level was decreased significantly compared with those treated with the scrambled siRNA control or to the cells attached to collagen IV (Fig. 6*B*). Phospho-p38 levels were similar in the Bit-1 knockdown cells and controls, suggesting that in HeLa cells Bit-1 does not impact the p38 signal transduction pathway (Fig. 6*C*)**.** These data support a role for Bit-1 in adhesion-dependent AKT activation in multiple cell types.

Bit-1 Activates NFκB Pathway—The PI3K/AKT pathway can activate NFκB and thereby up-regulate *bcl-2* transcription (25). Activated AKT induces increased phosphorylation of IκB that promotes IκB ubiquitination and subsequent proteolytic degradation. This results in NFκB release and translocation into the nucleus and enhanced NFKB-dependent transcription. Therefore, we examined whether forced expression of wild type Bit-1 promoted NFκB activity through IκB phosphorylation in HeLa cells attached to fibronectin in serum-free conditions. Indeed, expression of Bit-1 increased pFAK and pAKT levels (Fig. 7, *A* and *B*). Bit-1 expression also increased pIκB levels compared with vector controls (Fig. 7*C*). Bit-1 overexpression did not alter phospho or total p38 levels (Fig. 7*D*). We confirmed that Bit-1 is overexpressed in the cell lines used for these analyses (Fig. 7*E*). Thus, Bit-1 can promote cell survival in attached cells via a FAK-PI3K-AKT-NFκB pathway that up-regulates *bcl-2* expression.

Bit-1 Is in a Complex with FAK—Dominant negative FAK blocks Bit-1 mediated cell survival in attached cells, suggesting

[3] G. S. Griffiths, A. Leychenko, S. Reiter, and M. L. Matter, unpublished observations.

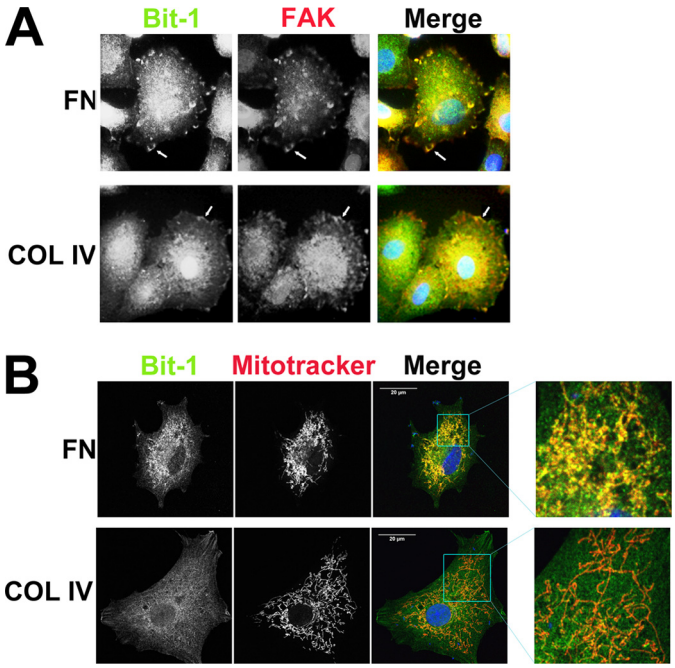

FIGURE 8. **Bit-1 co-localizes with FAK.** *A*, Bit-1 co-localizes with FAK at the plasma membrane in H9c2 myoblasts attached to fibronectin (*FN*) or to collagen IV (*COL IV*). Bit-1 and FAK co-localize in cells as examined by immunofluorescence microscopy at 63×. Co-localization of Bit-1 and FAK is displayed in *yellow* in the merged image. *Arrows* indicate co-localization. H9c2 cells were plated on fibronectin- or collagen IV-coated glass slides and allowed to spread for 1 h. Bit-1 was detected by immunostaining with an anti-Bit-1 antibody followed by a fluorescence 488-nm conjugated secondary. FAK was detected by immunostaining with an anti-FAK antibody followed by a fluorescence 594-nm conjugated secondary. Images were collected using a Zeiss Axiovert 200 M microscope. Data shown are representative of three independent experiments. *B*, a portion of Bit-1 co-localizes at the mitochondria upon attachment to collagen IV or fibronectin. Cells attached for 1 h to fibronectin or collagen IV were incubated with Mitotracker Red 594 nm prior to immunostaining with an anti-Bit-1 antibody followed by a fluorescence 488 nm conjugated secondary (63×). Images were collected using a Leica TCS SP5 confocal microscope. Data shown are representative of three independent experiments.

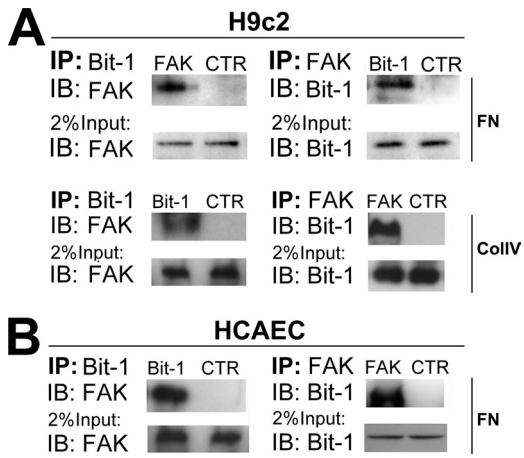

FIGURE 9. **Bit-1 associates with FAK.** *A*, Bit-1 and FAK co-immunoprecipitate. H9c2 cells plated on fibronectin (*FN*) or collagen IV (*ColIV*) were lysed and immunoprecipitated (*IP*) with anti-Bit-1 or anti-FAK conjugated IgG beads or IgG beads alone control, and immunoprecipitates were analyzed by immunoblotting (*IB*) with either an anti-Bit-1 or anti-FAK antibody. Lower immunoblots (*IB*) demonstrate 2% input of either FAK or Bit-1. Data shown are representative of three independent experiments. *B*, Bit-1 and FAK co-immunoprecipitate in normal endothelial cells. HCAECs plated on fibronectin were lysed and immunoprecipitated with anti-Bit-1- or anti-FAK-conjugated IgG beads or IgG beads alone control, and immunoprecipitations were analyzed by immunoblotting with either an anti-Bit-1 or anti-FAK antibody. Lower immunoblots demonstrate 2% input of either FAK or Bit-1. Data shown are representative of three independent experiments.

that Bit-1 acts upstream of FAK. We therefore examined whether Bit-1 was in a complex with FAK, as we had observed that in myoblast cell types (primary cardiomyocytes and H9c2 cells) Bit-1 was localized to the plasma membrane upon cell attachment. In H9c2 cells attached to fibronectin, Bit-1 co-localized with FAK at the plasma membrane (Fig. 8*A*). Examination of H9c2 cells attached to collagen IV demonstrated that Bit-1 co-localized with FAK at the plasma membrane similar to cells attached to fibronectin (Fig. 8*A*). Upon attachment to collagen IV or fibronectin a portion of Bit-1 also co-localized with the mitochondria (Fig. 8*B*), which is in agreement with previous findings (11).

To further test that Bit-1 and FAK are in a complex in adherent cells, we immunoprecipitated Bit-1 from H9c2 cell lysates of cells bound to fibronectin or collagen IV and examined for co-precipitation of FAK by immunoblotting. On both substrates, FAK was detected in Bit-1 immunoprecipitations, and in reciprocal co-immunoprecipitations, Bit-1 was detected in FAK immunoprecipitations (Fig. 9*A*), confirming that Bit-1 and FAK are in a complex in adherent H9c2 cells.

We next examined whether the Bit-1/FAK association occurred in normal endothelial cells. We immunoprecipitated Bit-1 from HCAEC cell lysates and examined for co-precipitation of FAK by immunoblotting. FAK was detected in Bit-1 immunoprecipitations, and in reciprocal co-immunoprecipitations, Bit-1 was detected in FAK immunoprecipitations (Fig. 9*B*). This finding confirms that Bit-1 and FAK are in a complex in adherent normal cells.

DISCUSSION

We report that in attached cells Bit-1 protects from serum deprivation-mediated apoptosis and staurosporine-induced mitochondrial apoptosis by up-regulating NFκB activity and subsequently *bcl-2* gene transcription. Protection from apoptosis under serum-free conditions correlated with increased *bcl-2* transcription and Bcl-2 protein expression. Bit-1-mediated up-regulation of NFκB is dependent on activation of FAK and PI3K. We thereby define a pathway whereby Bit-1 enhances integrin $\alpha5\beta1$ activation of FAK followed by activation of PI3K, AKT, and NFκB leading to the elevation of Bcl-2 levels and consequent cell survival. Based on the data reported here, we have refined our model of integrin-mediated Bit-1 function (Fig. 10). When cells lose their attachment to the underlying ECM and no integrin is ligated, Bit-1 is released from mitochondria into the cytoplasm where it binds to the proapoptotic AES-TLE complex and enhances anoikis (11). This may be due to phosphorylation of Bit-1 by other signaling molecules such as PKD1 (13). However in cells attached to fibronectin, Bit-1 works in conjunction with a FAK/PI3K/NFκB signaling pathway and elevates *bcl-2* transcription to promote cell survival. The findings reveal a novel function of Bit-1 as a mediator of integrin-mediated cell survival.

Bit-1 conditional knock-out mice are smaller than wild type littermates, develop a runting syndrome, and die within the first 2 weeks of life (14). We report here that knockdown of endog-

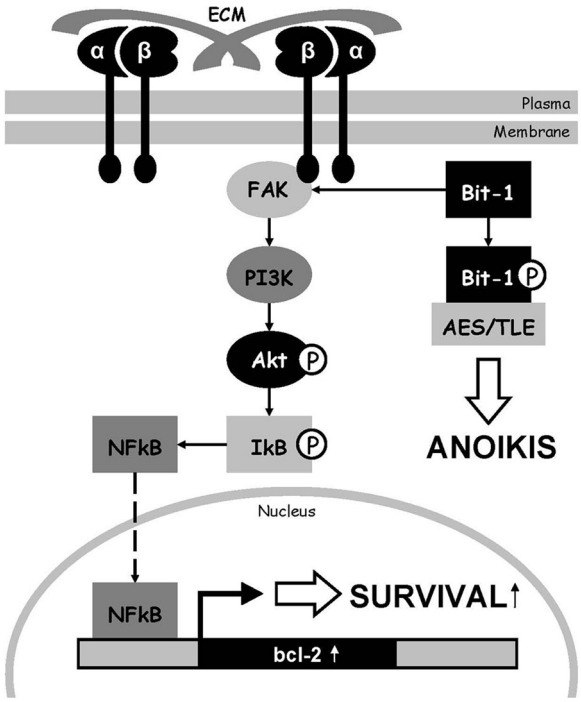

FIGURE 10. **Integrin-regulated Bit-1 survival pathway in attached cells.** Cell attachment to the ECM through the $\alpha5\beta1$ integrin results in the activation of a signal transduction pathway that promotes cell survival. This pathway starts with integrin-mediated activation of FAK followed by activation of PI3K and AKT and consequently enhanced *bcl-2* transcription that correlates with cell survival. Bit-1 acts on this pathway upstream of FAK and PI3K. Bit-1 increases pIκB levels, which promotes the translocation of NFκB into the nucleus where it induces *bcl-2* transcription and correlates with cell survival. When cells are placed in suspension and no integrin is bound, the integrin/Bit-1/FAK/PI3K/NFκB/bcl-2 survival pathway is not activated, and Bit-1 interacts with the proapoptotic AES-TLE complex to enhance anoikis.

enous Bit-1 levels enhanced apoptosis mediated by serum deprivation in cells attached to fibronectin. Moreover, re-expression of Bit-1 in these cells abrogated this effect and correlated with increased cell survival. Loss of Bit-1 also enhanced staurosporine-induced apoptosis in attached cells. This is in agreement with Kairouz-Wahbe and colleagues (14) who reported that Bit-1 KO mouse embryonic fibroblasts are more sensitive to staurosporine-mediated apoptosis compared with wild type mouse embryonic fibroblasts. We found that caspase-3 activation was increased significantly in staurosporine-treated cells lacking Bit-1. In addition, staurosporine treatment in these cells enhanced apoptotic DNA fragmentation as determined by Apoptag analysis. Our studies have found similar results in a variety of cell types, suggesting that this function of Bit-1 in adherent cells is conserved. These data suggest a survival role for Bit-1 in attached cells and suggest that the runted growth evident in the KO mice may be due in part to poor cell viability in some tissues.

Our data indicate that Bit-1-mediated regulation of *bcl-2* transcription requires FAK. Expression of a dominant negative form, FRNK, inhibited Bit-1-mediated up-regulation of *bcl-2* transcription. This finding suggests that Bit-1 activity is upstream of FAK. FAK is important for the survival of anchorage-dependent cells and can activate a number of prosurvival signaling pathways. FAK promotes up-regulation of *bcl-2* in cells that signal through the $\alpha5\beta1$ integrin but not in cells that signal via $\alpha v\beta1$ (3). Activation of this integrin/FAK/PI3K/Bcl-2 prosurvival pathway promotes NFκB mediated transcription (26), and FAK can thereby promote cell survival via NFκB (27). Upon integrin ligation, Bit-1 may signal through FAK to increase *bcl-2* transcription and protein levels to promote cell survival. In myoblasts and endothelial cells, we found that, upon attachment to the ECM, Bit-1 co-localized with FAK at the plasma membrane and that these proteins associated in a complex in reciprocal co-immunoprecipitations. Based on these data, we posit that a functional association is required between FAK and Bit-1 for Bit-1-mediated survival signaling. Interestingly, Bit-1 and FAK also associated on collagen IV, suggesting that the signaling complex requires more than the physical association. Bit-1 phosphorylation by other proteins also may be involved (13). Integrin attachment promotes cell survival through the PI3K pathway via FAK, and both $\alpha5\beta1$ and $\alpha v\beta3$ pro-survival signals are through PI3K (3). Our data indicate that Bit-1 regulation of *bcl-2* transcription also occurs through PI3K. Therefore, in attached cells, Bit-1 signals through an integrin-mediated FAK/PI3K/AKT/Bcl-2 survival pathway.

These results suggest the hypothesis that Bit-1 may be the integrin effector protein that targets integrin signals to the NFκB survival pathway. ERK also activates the anti-apoptotic NFκB pathway via RSK by phosphorylating the NFκB inhibitor IκB and inducing its degradation (28–31); however, Bit-1 does not appear to activate this pathway, and indeed, Bit-1 expression decreases Erk activity (14). Thus, Bit-1 is unlikely to promote cell survival through the ERK1/2 pathway. Thus, our data point to Bit-1 activation of a FAK to PI3K to NFκB pathway leading to increased *bcl-2* transcription and subsequent increased cell survival in attached cells. However, we cannot completely exclude the possibility of an integrin-independent mechanism that may also effect Bit-1 survival signals.

AKT-dependent regulation of NFκB and Bcl-2 expression is not limited to integrins as both growth factors and cytokines can also activate these pathways. IL-1 activates NFκB through the PI3K/AKT/mTOR pathway (32), and osteoclast survival occurs through a TGF-β induced TAK1/MEK-mediated AKT activation and NFκB signaling cascade (33, 34). Vascular endothelial growth factor also has been shown to activate PI3K (35), elevate Bcl-2 expression (35, 36), and to enhance NFκB mediated survival (37). It will be important to determine whether these other prosurvival signal transduction pathways converge on Bit-1 as Bit-1 may act as a key molecular switch at the nexus of cellular life and death.

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