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$\alpha_5\beta_1$ Integrin Stimulates Bcl-2 Expression and Cell Survival Through Akt, Focal Adhesion Kinase, and Ca^{2+}/Calmodulin-Dependent Protein Kinase IV

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Abstract CHO cells expressing $\alpha_5\beta_1$ integrin are more resistant to apoptosis and express more Bcl-2 than the same cells engineered to express $\alpha_v\beta_1$ or cytoplasmically truncated $\alpha_5\Delta c\beta_1$ integrin as their main fibronectin receptor. The Bcl-2 up-regulation by $\alpha_5\beta_1$ is mediated, at least in part, by the focal adhesion kinase (FAK) and phosphatidylinositol-3 kinase (PI3K)/Akt pathways. Here, we show that integrin-mediated activation of Ca^{2+}/calmodulin-dependent protein kinase (CaMK) IV, and the NF-κB and CREB transcription factors also enhance the integrin-dependent regulation of Bcl-2 expression in the $\alpha_5\beta_1$cells. A forkhead transcription factor, which is inactivated by Akt, blocked Bcl-2 expression. The FAK pathway was found to be defective in both the $\alpha_v\beta_1$ and $\alpha_5\Delta c\beta_1$ cells. These cell lines differed from one another in two Bcl-2-regulating pathways: adhesion through $\alpha_v\beta_1$ failed to activate Akt, allowing forkhead to suppress Bcl-2 transcription, whereas $\alpha_5\Delta c\beta_1$ did not activate NF-κB and CREB, presumably because CaMK IV was not activated. Our results indicate that three pathways, the FAK, PI3K/Akt, and CaMK IV mediate the survival-supporting activity of $\alpha_5\beta_1$ integrin. J. Cell. Biochem. 95: 1214–1223, 2005. © 2005 Wiley-Liss, Inc.

Key words: Akt; Bcl-2; CaMK; cell adhesion; fibronectin; integrin

Integrins are the main cellular receptors for attachment to the extracellular matrix. In response to the matrix attachment, integrins activate intracellular signaling pathways, and these signals play a role in the control of cell cycle and survival [Giancotti and Ruoslahti, 1999]. Loss of cell attachment to the matrix causes apoptosis; this process is referred to as "anoikis" [Meredith et al., 1993; Frisch and Francis, 1994; Frisch and Screaton, 2001]. Anoikis may help maintain the integrity of tissues, as it would prevent detached cells from establishing themselves at inappropriate locations. Relative resistance of malignant cells to anoikis is likely to play a role in invasion and metastasis.

Different types of cells depend on different integrins for survival. For example, the $\alpha_5\beta_1$ integrin promotes survival of many cell types such as epithelial cells, endothelial cells, and fibroblasts [Zhang et al., 1995; Wary et al., 1996; Lee and Juliano, 2000]. The $\alpha_v\beta_3$ integrin is particularly important for survival of angiogenic endothelial cells [Brooks et al., 1994a,b]. The $\alpha_4\beta_1$ integrin mediates B lymphocyte survival [Koopman et al., 1994]. Studies on integrin knockout mice also indicate specific functions for the various integrins, some of which may relate to survival. The phenotypes of these mice range from major developmental abnormalities (α_4, α_5, α_v, β_1, β_8) to defects in leukocyte function (α_L, β_2, β_7), hemostasis (α_{IIb}, α_2, β_3) and angiogenesis (α_1, β_3), and abnormal inflammatory responses (β_6) [Hynes, 2002].

Akt activity is central to cell survival; Akt phosphorylates and thereby inactivates the

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pro-apoptotic proteins, Bad and caspase-9 [Datta et al., 1999], and elevates the expression of the anti-apoptotic protein, Bcl-2 [Matter and Ruoslahti, 2001]. Integrin-mediated cell attachment activates Akt through the phosphatidylinositol-3 kinase (PI3K), promoting cell survival [Khwaja et al., 1997; King et al., 1997; Lee and Juliano, 2000; Tian et al., 2002]. Among the other major pathways of integrin signaling, focal adhesion kinase (FAK) is known to convey survival signals from the extracellular matrix [Frisch et al., 1996; Hungerford et al., 1996; Ilic et al., 1998; Matter and Ruoslahti, 2001]. FAK may promote survival through the PI3K/Akt pathway [Chen and Guan, 1994; Tamura et al., 1999]. However, inhibition of the pro-apoptotic activities of p53 may be more central to the survival effects of FAK [Stromblad et al., 1996; Ilic et al., 1998].

Growing evidence indicates that the integrin-mediated signaling pathways, including those controlling anoikis, can be specific to individual integrins [Giancotti, 2000]. For example, a subset of integrins consisting of $\alpha_1\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ can recruit the adaptor protein Shc and use that to activate Ras signaling [Wary et al., 1996]. Earlier studies from our laboratory have shown that the adhesion mediated by the $\alpha_5\beta_1$ integrin supports the survival of cells stressed serum withdrawal, whereas another fibronectin receptor, the $\alpha_v\beta_1$ integrin, is inefficient in this regard [Zhang et al., 1995]. The survival signal is at least partially mediated by transcriptional up-regulation of the anti-apoptotic protein Bcl-2, as cell attachment does not up-regulate Bcl-2 in the $\alpha_v\beta_1$ cells. Earlier studies have also shown that CHO cells expressing $\alpha_5\beta_1$ integrin with a truncated α_5 subunit cytoplasmic domain ($\alpha_5\Delta c\beta_1$ cells) are similar to the $\alpha_v\beta_1$ cells, in that they are prone to apoptosis and fail to up-regulate Bcl-2 upon attachment. Integrin dependence of these effects was shown by the use of alternative substrates and blocking antibodies against individual integrins [Zhang et al., 1995; Matter and Ruoslahti, 2001].

Here, we show that the $\alpha_v\beta_1$ and $\alpha_5\Delta c\beta_1$ CHO cells have a different defect in Bcl-2-regulating integrin signaling pathways. We make use of this observation to dissect the signaling pathways responsible for integrin-mediated up-regulation of Bcl-2. The results indicate that efficient Bcl-2 expression in cells attached to fibronectin requires the cooperation of three distinct pathways. We also identify the transcriptional regulators, which are likely to control the integrin regulation of Bcl-2 expression.

MATERIALS AND METHODS

Cells

An $\alpha_5\beta_1$-deficient CHO cell line (B2), which expresses ~2% of wild type $\alpha_5\beta_1$ [Schreiner et al., 1989], was from Dr. Rudolf Juliano (University of North Carolina, Chapel Hill, NC). The CHO-B2/$\alpha_5\beta_1$ and CHO-B2/$\alpha_v\beta_1$ cells that express high levels of α_5 and α_v were generated by introducing cDNAs coding for the human α_5 and α_v integrin subunits to the CHO-B2 cells, respectively [Bauer et al., 1993; Zhang et al., 1993]. No $\alpha_v\beta_1$ was detected in the parental B2 cell line, or in the CHO-B2/$\alpha_5\beta_1$ cell line [Zhang et al., 1993]. The CHO-B2/$\alpha_5\Delta c\beta_1$ cells were generated by transfecting a truncated α_5 construct that lacked the cytoplasmic domain into the CHO-B2 cells [Bauer et al., 1993]. All cells were maintained in a modified Eagle's medium (α-MEM) supplemented with 10% fetal bovine serum, glutamine/penicillin-streptomycin, and 250 μg/ml of G418.

Plasmid Constructs

The Bcl-2 promoter construct containing −3.7 kb of human Bcl-2 gene fused to a luciferase gene was obtained from Dr. John C. Reed (Burnham Institute). The pCMV-Flag-p65 expression vector was from Dr. Albert S. Baldwin, Jr. (University of North Carolina), the pRSV-CREB vector from Dr. Marc Montminy (Salk Institute, San Diego, CA), the pSG5-CaMK IV K75E vector from Dr. Anthony R. Means (Duke University, Durham, NC), and the pECE-HA-FKHRL1 vector with triple mutations (T32A, S253A, and S315A) from Dr. Michael Greenberg (Harvard Medical School, Boston, MA). The pNF-κB-Luc and pCRE-Luc reporter constructs were purchased from Clontech (Palo Alto, CA).

Antibodies and Reagents

Mouse monoclonal anti-Bcl-2 antibody was purchased from BD Biosciences (San Jose, CA), and rabbit polyclonal antibodies against phospho-Akt (Ser473), Akt, phospho-FKHR (Ser256), and FKHR were from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-phospho-FAK (Tyr397) and anti-FAK antibodies were from Biosource (Camarillo, CA), rabbit polyclonal anti-Bax antibody from Santa Cruz (Santa Cruz, CA), and mouse monoclonal anti-

actin antibody from Chemicon (Temecula, CA). LY294002 was purchased from Cell Signaling Technology. The CaMK inhibitors KN62 {1-[*N*, *O*-bis-(5-Isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine} and KN93 {2-[*N*-(2-hydroxyethyl)]-*N*-(4-methoxybenzenesulfonyl)} amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine)}, and the Ca^{2+} chelators BAPTA [1, 2-bis(*o*-Aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid] and BAPTA/AM, cell-permeable form of BAPTA, were from Calbiochem (San Diego, CA).

Immunoblotting

Cells were plated on culture dishes coated with 10 μg/ml of fibronectin or native type I collagen overnight at 4°C or and then incubated for varying periods of time in serum-free medium containing 0.1% bovine serum albumin. The number of plated cells was at 6×10^6 cells/75 mm bacterial culture dishes or 1×10^6 cells/well in 6-well culture dishes. Cells were lysed with a lysis buffer containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and protease inhibitors/phosphatase inhibitors. Equal amounts of cell lysates were resolved on 4%–20% SDS–polyacrylamide gels. After electrophoresis, proteins were transferred to Immobilon-P nylon membrane (Millipore, Billerica, MA) and immunoblotted. Immunoblots were developed by ECL-plus (Amersham Biosciences, Piscataway, NJ).

Annexin V Apoptosis Assays

Cells were plated onto culture dishes at 1×10^6 cells/well in 6-well plates coated with 10 μg/ml of fibronectin and then incubated in serum-free medium containing 0.1% bovine serum albumin for 24 h. After incubation, the cells were harvested with trypsin, washed with serum-containing media, and then resuspended in binding buffer. Cells were incubated with annexin V-FITC (a final concentration of 0.5 μg/ml) according to the manufacturer's protocol (Clontech) and analyzed by flow cytometry. More than 1×10^4 events were counted for each sample.

Transfection and Luciferase Assays

The day before transfection, cells were plated on culture dishes at 2×10^5 cells/well in 24-well plates or 1×10^6 cells/well in 6-well plates. Cells were transfected with various luciferase constructs and expression vectors (a total 0.8 μg DNA per well in a 24-well plate and 4 μg DNA per well in a 6-well plate) for 24 h using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Luciferase activity was measured by dual-luciferase reporter assay system (Promega, Madison, WI) in a 96-well plate luminometer. The measured luciferase activity was normalized for transfection efficiency by renilla luciferase internal control.

RESULTS

$\alpha_5\beta_1$ Cells Are More Resistant to Stress-Induced Apoptosis Than $\alpha_v\beta_1$ and $\alpha_5\Delta c\beta_1$ Cells, and Express More Bcl-2 Protein

Annexin V staining showed that $\alpha_5\beta_1$ cells plated on fibronectin, which is a ligand for the $\alpha_5\beta_1$ and $\alpha_v\beta_1$ integrins, and stressed by incubating in serum-free medium exhibited 40%–50% fewer apoptotic cells than $\alpha_v\beta_1$ or $\alpha_5\Delta c\beta_1$ cells (Fig. 1, open bars). The difference was even more pronounced, up to fourfold, when a low concentration of the apoptosis-inducing agent, staurosporine, was used as an additional stress-inducing agent (Fig. 1A, filled bars). These results are in agreement with earlier observations [Zhang et al., 1995; Matter and Ruoslahti, 2001], showing that $\alpha_5\beta_1$ integrin-mediated cell attachment is associated with protection against apoptosis.

We also found that fibronectin attachment of $\alpha_5\beta_1$ cells up-regulates Bcl-2 expression, and that PI3K plays an important role in it. Serum-starved $\alpha_5\beta_1$ cells plated on fibronectin expressed more Bcl-2 protein than in $\alpha_v\beta_1$ and $\alpha_5\Delta c\beta_1$ cells, and the PI3K inhibitor LY294002 down-regulated Bcl-2 protein induced by $\alpha_5\beta_1$ cells (Fig. 1B). The levels of the pro-apoptotic protein, Bax, were similar among these cell lines (Fig. 1B). Comparison of Bcl-2 protein levels in cells plated onto fibronectin or collagen, which is not a ligand for the $\alpha_5\beta_1$ and $\alpha_v\beta_1$ integrins, showed that only fibronectin caused up-regulation of Bcl-2 (Fig. 1C).

PI3K/Akt Pathway Is Activated in Attached $\alpha_5\beta_1$ and $\alpha_5\Delta c\beta_1$ Cells but not in $\alpha_v\beta_1$ Cells

Akt is activated by phosphorylation at serine 473 and threonine 308 [Datta et al., 1999]. We used phosphorylation-specific anti-Akt antibodies to examine Akt activation. Abundant phospho-Akt (serine 473) was found in the $\alpha_5\beta_1$

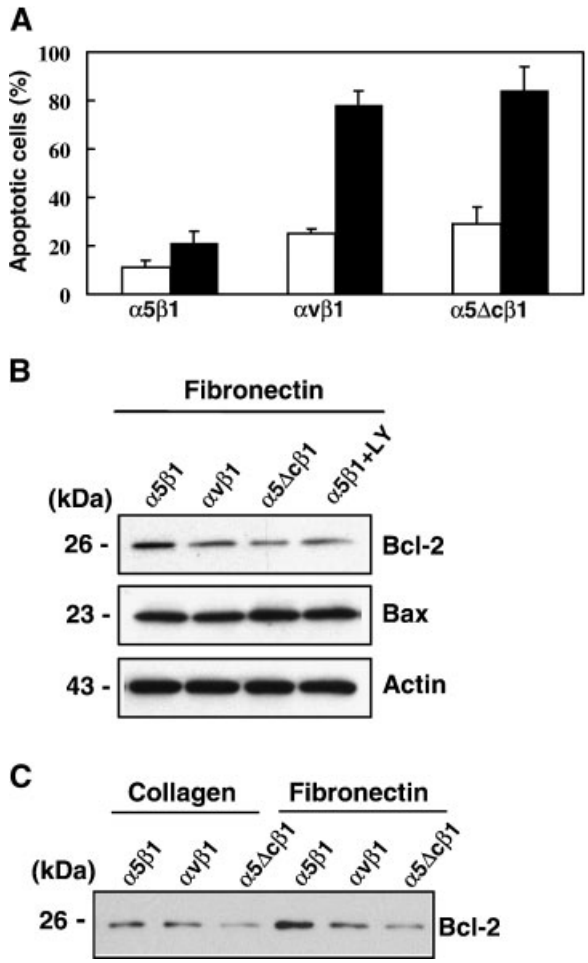

Fig. 1. Apoptosis and Bcl-2 expression in CHO cells expressing different fibronectin-binding integrins. **A**: $\alpha_5\beta_1$ cells are more resistant to serum depletion and staurosporine-induced apoptosis than cells expressing the $\alpha_v\beta_1$ or $\alpha_5\Delta c\beta_1$ integrin. Cells were plated onto fibronectin-coated dishes (10^6 cells/well in 6-well plates) and incubated in serum-free medium with (filled bars) or without (open bars) 2 nM staurosporine. After a 24-h incubation, the cells were analyzed for annexin V binding using flow cytometry. Data represent the percentage (as mean ± SD from three experiments) of annexin V positive cells in each condition. **B** and **C**: $\alpha_5\beta_1$ cells express Bcl-2 protein at higher levels than $\alpha_v\beta_1$ or $\alpha_5\Delta c\beta_1$ cells. Cells (6×10^6 cells/75 mm dish) were plated on fibronectin-coated dishes or native type I collagen, and incubated in serum-free medium with or without 50 μM LY294002 (LY). After a 24-h incubation, cell lysates were prepared and subjected to immunoblot analysis with the indicated antibodies. A representative experiment is shown in each case.

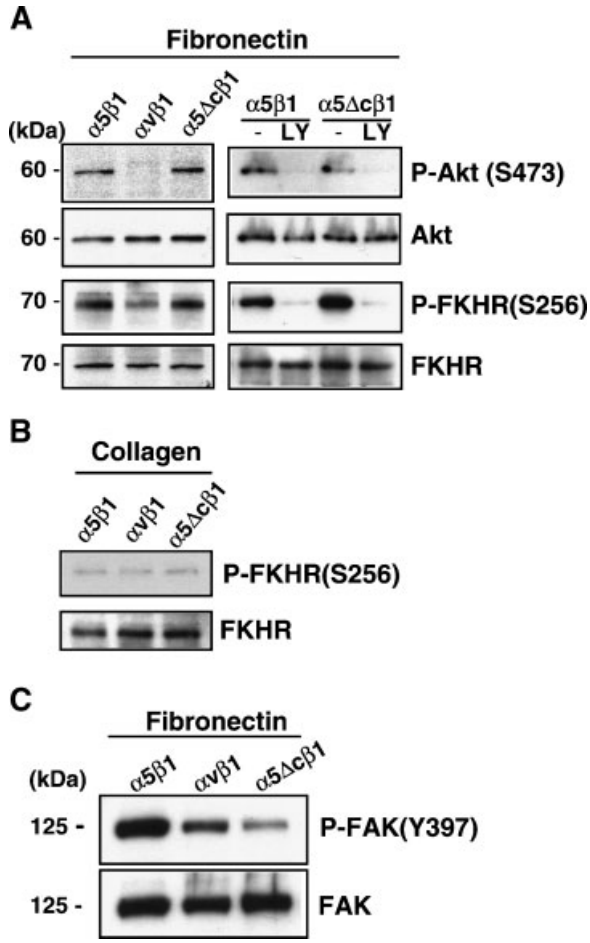

Fig. 2. Phosphorylation of Akt, forkhead transcription factors, and FAK. Cells (6×10^6 cells/75 mm dish) were plated onto dishes coated with fibronectin (**A** and **C**) or native type I collagen (**B**), and incubated in serum-free medium with or without 50 μM LY294002 (LY). After a 4-h incubation, cell lysates were prepared and subjected to immunoblot analysis with the indicated antibodies. These experiments were repeated at least three times with similar results.

and $\alpha_5\Delta c\beta_1$ cells, while the $\alpha_v\beta_1$ cells contained mostly non-phosphorylated Akt (Fig. 2A). The total protein levels of Akt were similar among these cells. Similar results were obtained with antibodies against Akt phosphorylated at threonine 308 (data not shown). The phosphorylation level of Akt in $\alpha_5\beta_1$ and $\alpha_5\Delta c\beta1$ cells was decreased by the inhibition of PI3K with LY294002 (Fig. 2A), indicating that PI3K was responsible for the Akt activation.

Akt inhibits apoptosis by phosphorylating a number of cellular proteins [Datta et al., 1999]. The forkhead transcription factors encompass one group of Akt substrate proteins. Three members of the forkhead family transcription factor (FKHR, FKHRL1, and AFX) have been identified in mammalian cells [Brunet et al., 1999; Datta et al., 1999]. Each of our test cell lines expressed FKHR at a similar level, but a greater portion of FKHR was phosphorylated (inactive) in the $\alpha_5\beta_1$ and $\alpha_5\Delta c\beta_1$ cells than in the $\alpha_v\beta_1$ cells (Fig. 2A). Treatment with LY294002 reduced FKHR phosphorylation. A similar

pattern of phosphorylation was observed in FKHRL1, which is also expressed by the CHO cells (not shown). FKHR phosphorylation was not appreciably enhanced in any of the CHO lines on collagen (Fig. 2B). Non-integrin dependent attachment to poly-L-lysine also did not induce FKHR phosphorylation, or Akt activation (not shown). These results indicate that the $\alpha_5\beta_1$ integrin, but not $\alpha_v\beta_1$ or collagen-binding integrins, activates the Akt pathway in a manner that does not require the α_5 subunit cytoplasmic domain.

FAK Activation Depends on Integrin Type

Akt activation may explain the up-regulated Bcl-2 expression and enhanced survival of $\alpha_5\beta_1$ cells on fibronectin compared to the $\alpha_v\beta_1$ cells. However, it could not explain these same properties in $\alpha_5\Delta c\beta_1$ cells, as the Akt activation level in these cells was similar to that in $\alpha_5\beta_1$ cells. Rather, it seemed that additional signaling pathways may be involved in the $\alpha_5\beta_1$ integrin-induced Bcl-2 expression.

We first examined the involvement of FAK, since previous studies reported that FAK plays a role in cell survival [Frisch et al., 1996; Ilic et al., 1998; Almeida et al., 2000; Sonoda et al., 2000] and in Bcl-2 expression [Matter and Ruoslahti, 2001]. More active FAK, measured by detecting phosphorylation at tyrosine 397 [Schaller et al., 1994], was found in $\alpha_5\beta_1$ cells than in $\alpha_v\beta_1$ and $\alpha_5\Delta c\beta_1$ cells (Fig. 2C). The reduction of FAK activation was less pronounced in the $\alpha_v\beta_1$ cells than in $\alpha_5\Delta c\beta_1$. Phosphorylation at tyrosine 577 residue, which is an activation-related residue in the kinase domain of FAK [Calalb et al., 1995], was also enhanced in the $\alpha_5\beta_1$ cells (not shown). These results show that the activity of the FAK pathway is reduced in the $\alpha_5\Delta c\beta_1$ cells when compared to the $\alpha_5\beta_1$ cells. This is also true of the $\alpha_v\beta_1$ cells, but to a lesser extent. These results indicate that integrin type-dependent changes in FAK activation contribute to the differences in Bcl-2 expression and cell survival in the different cell lines, but are not solely responsible for those differences.

CaMK IV Regulates Bcl-2 Expression and Cell Survival in $\alpha_5\beta_1$ Cells

The $\alpha_5\beta_1$ and $\alpha_v\beta_1$ cells attach to and spread well on fibronectin, but the $\alpha_5\Delta c\beta_1$ cells do so less well, despite similar expression levels of the α_5 subunit at the cell surface [Bauer et al., 1993]. Cell spreading stretches the nucleus and increases nuclear [Ca^{2+}] with subsequent activation of Ca^{2+}/calmodulin-dependent protein kinase (CaMK) IV [Itano et al., 2003]. We surmised that CaMK IV activated through this pathway might mediate the regulation of Bcl-2 expression. We found that chelating intracellular Ca^{2+} (including nuclear Ca^{2+}) with the cell-permeable chelator BAPTA/AM reduced Bcl-2 expression in the $\alpha_5\beta_1$ cells, whereas chelating extracellular Ca^{2+} with BAPTA had no effect (Fig. 3A). Inhibiting CaMK IV with KN93 and KN62, two cell-permeable CaMK inhibitors, did the same. Furthermore, transfection of the $\alpha_5\beta_1$ cells with the dominant negative CaMK IV K75E down-regulated Bcl-2 expression (Fig. 3A). In agreement with the changes in Bcl-2 expression, chelating intracellular Ca^{2+} or inhibiting CaMK IV increased the apoptosis rate of the $\alpha_5\beta_1$ cells by four to five-fold (Fig. 3B). Thus, CaMK IV appears to play a role in Bcl-2 expression and regulates cell survival.

NF-κB, CREB, and Forkhead Transcription Factors in $\alpha_5\beta_1$ Integrin-Mediated Bcl-2 Expression and Cell Survival

NF-κB and CREB transcription factors have been shown to induce Bcl-2 mRNA and protein expression [Pugazhenthi et al., 1999, 2000; Kurland et al., 2001; Heckman et al., 2002]. Both NF-κB and CREB can be activated by Akt and CaMK [Sun et al., 1996; Jang et al., 2001; Redmond et al., 2002]. Moreover, NF-κB is activated and the cytoplasmic levels of IκB protein are reduced upon the adhesion of cells to fibronectin [Bearz et al., 1998; Klein et al., 2002]. We therefore examined the role of these transcription factors in the $\alpha_5\beta_1$ integrin-mediated Bcl-2 expression. We transfected cells with NF-κB and CRE reporter constructs that contain the consensus NF-κB and CRE binding site fused to the luciferase gene, and plated the cells onto fibronectin-coated dishes. The $\alpha_5\beta_1$ and $\alpha_v\beta_1$ cells showed similar levels of intracellular NF-κB and CREB activity, while 40% of NF-κB and 30% of CREB activity was seen in the $\alpha_5\Delta c\beta_1$ cells, compared to the $\alpha_5\beta_1$ cells (Fig. 4A,B). Blockade of the PI3K/Akt pathway with LY294002 in $\alpha_5\beta_1$ cells, or CaMK with KN93 in $\alpha_5\beta_1$ and $\alpha_v\beta_1$ cells, reduced the activity of both the NF-κB and CRE reporter by approximately 50%. The activity of NF-κB or CREB was not induced on collagen in any of the

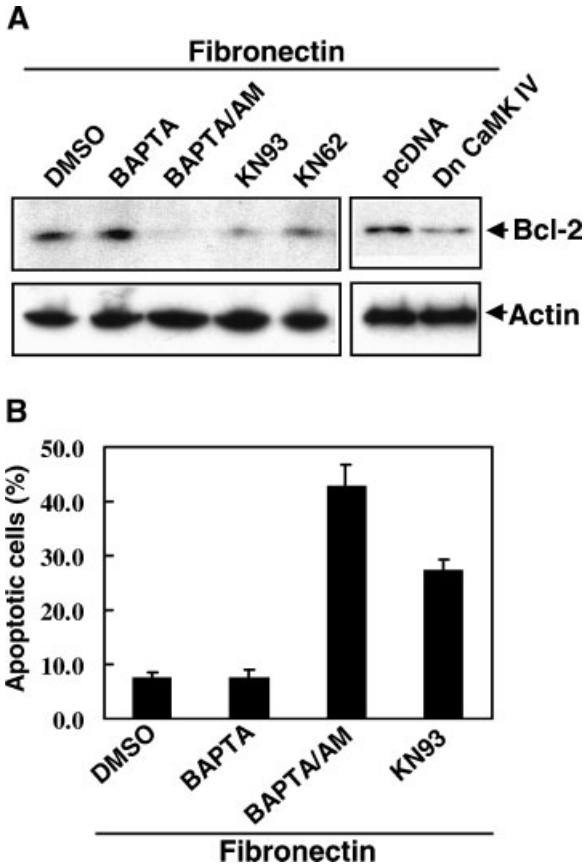

Fig. 3. Inhibition of CaMK and chelation of intracellular Ca^{2+} down-regulate Bcl-2 expression and cell survival. **A** (**left panel**): $\alpha_5\beta_1$ cells were plated onto fibronectin-coated dishes (10^6 cells/well in 6-well plates) in serum-free medium. The cells were incubated either with the CaMK inhibitor KN93 (10 μM) or KN62 (20 μM), or with the Ca^{2+} chelator BAPTA (0.5 mM) or its cell-permeable variant, BAPTA/AM (50 μM), or the vehicle (0.1% DMSO) for 24 h. **Right panel**: $\alpha_5\beta_1$ cells were plated on culture dishes (10^6 cells/well in 6-well plates), transfected with the dominant negative CaMK IV K75E or with control vector, and then incubated with serum-free medium for 24 h. After the incubation, cell lysates were prepared and subjected to immunoblot analysis using the indicated antibodies. A representative experiment out of three is shown. **B**: $\alpha_5\beta_1$ cells were plated onto fibronectin-coated dishes (10^6 cells/well in 6-well plates) and incubated with the indicated reagents. After a 24-h incubation, cells were analyzed for annexin V binding using flow cytometry. Data represent the percentage (as mean ± SD from three experiments) of annexin V-positive cells in each condition.

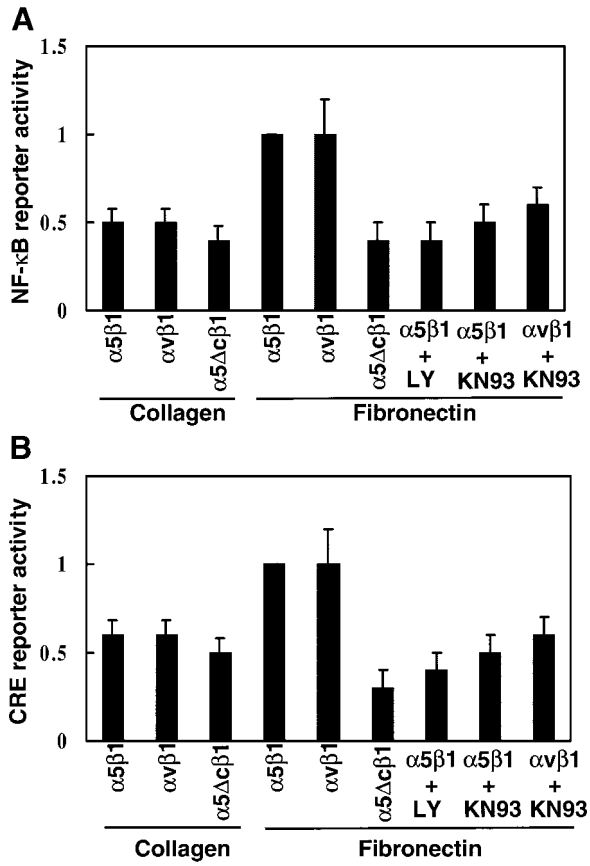

Fig. 4. NF-κB and CREB transcriptional activity. Cells (2×10^5 cells/well in 24-well plates) were transfected with NF-κB (**A**) or CRE luciferase reporter (**B**). After transfection, the cells were harvested, replated onto fibronectin-coated or collagen-coated dishes, and incubated with serum-free medium containing 50 μM of LY294002 (LY) or 10 μM of KN93. After a 24-h incubation, cell lysates were prepared and subjected to dual luciferase assay. Shown is luciferase activity (as mean ± SD from five independent experiments) relative to the activity in $\alpha_5\beta_1$ cells plated on fibronectin, which was made 1.0.

cell lines; the activity levels were consistently about 50% of those seen in $\alpha_5\beta_1$ cells plated on fibronectin (Fig. 4A,B).

To examine the regulation of Bcl-2 expression and cell survival by NF-κB and CREB, cells were transiently transfected with expression vectors for each factor and plated on fibronectin-coated dishes. Both NF-κB p65 subunit and CREB caused a four-fold enhancement of Bcl-2 promoter activity (Fig. 5A). Bcl-2 protein levels were also increased (Fig. 5B). Transfection of a constitutively active form of the forkhead transcription factor FKHRL1 decreased Bcl-2 promoter activity by 50% and concomitantly reduced Bcl-2 protein levels (Fig. 5A,B). In agreement with the changes in Bcl-2 levels, over-expression of CREB inhibited apoptosis, whereas FKHRL1 increased it (Fig. 5C). These results implicate NF-κB and CREB as the proximal mediators of the $\alpha_5\beta_1$ effect on Bcl-2 expression and cell survival, while forkhead serves as a negative regulator of the system.

DISCUSSION

Cell attachment mediated by the $\alpha_5\beta_1$ integrin supports cell survival and up-regulates

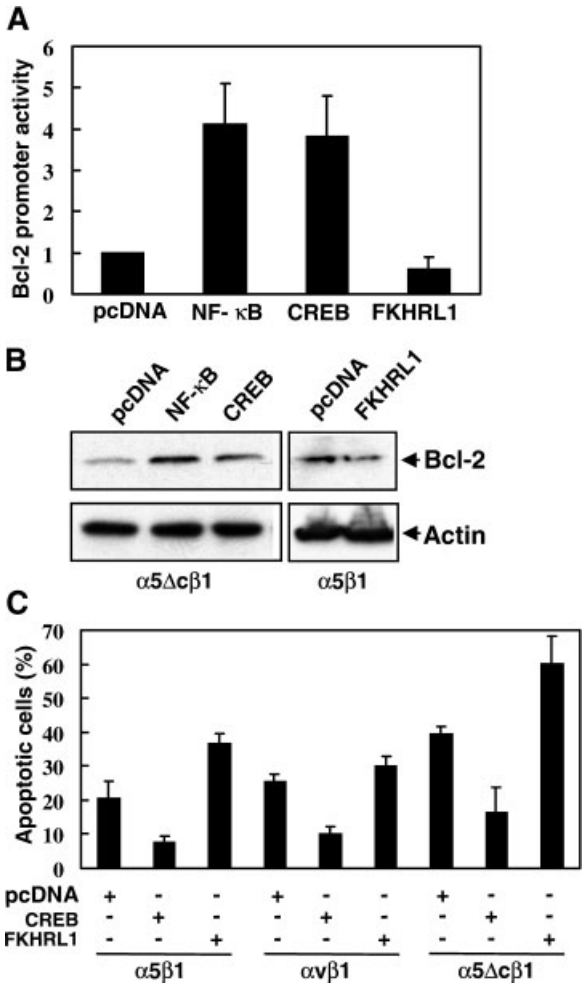

Fig. 5. NF-κB, CREB, and forkhead transcription factors regulate Bcl-2 expression and cell survival. **A**: $\alpha_5\beta_1$ cells (2×10^5 cells/well in 24-well plates) were transfected with Bcl-2 promoter-luciferase construct and the indicated expression vector. After transfection, cells were incubated in serum-free medium for 24 h and then subjected to dual luciferase assay. Data represent luciferase activity (as mean ± SD from five experiments) relative to the activity in cells transfected with the control vector (designated as 1.0). **B**: $\alpha_5\Delta c\beta_1$ cells (**left panel**) and $\alpha_5\beta_1$ cells (**right panel**) at a density of 10^6 cells/well in 6-well plates were transfected with the indicated expression vectors. After transfection, the cells were replated onto fibronectin-coated dishes and incubated in serum-free medium for 24 h, and then subjected to immunoblot analysis. **C**: Cells expressing different integrins (10^6 cells/well in 6-well plates) were transfected with expression vectors as indicated. After transfection, cells were replated onto fibronectin-coated dishes, incubated in serum-free medium for 24 h, and analyzed for annexin V binding. Data represent the percentage (as mean ± SD from three experiments) of annexin V positive cells in each condition.

Bcl-2 expression, whereas attachment mediated by the $\alpha_v\beta_1$ integrin, or by a mutant $\alpha_5\beta_1$ integrin with a truncated α_5 subunit cytoplasmic tail ($\alpha_5\Delta c\beta_1$), does not. We show here that the failure to induce Bcl-2 by the $\alpha_v\beta_1$ cells is in the Akt pathway, whereas a CaMK pathway is at fault in the $\alpha_5\Delta c\beta_1$ cells. Each of these pathways, as well as FAK activation, contributes to the integrin-dependent regulation of Bcl-2. They coordinately activate the positive transcription factors NF-κB and CREB, and suppress the negative transcriptional regulator forkhead.

Akt activity is critical for the survival of cells, including the CHO cells. Akt phosphorylates, and thereby inactivates, the pro-apoptotic proteins Bad, caspase 9 and forkhead, while phosphorylation by Akt activates NF-κB and CREB [Datta et al., 1999]. Integrin-mediated cell attachment activates Akt through PI3K, promoting cell survival [Khwaja et al., 1997; Lee and Juliano, 2000; Tian et al., 2002]. Our laboratory has previously shown that cell attachment mediated by the $\alpha_5\beta_1$ integrin supports cell survival and up-regulates Bcl-2 expression, and that constitutively activated Akt can substitute for $\alpha_5\beta_1$, whereas dominant negative Akt negates the $\alpha_5\beta_1$ effect [Matter and Ruoslahti, 2001]. The present data show that of the two low Bcl-2-expressor cell lines, the $\alpha_v\beta_1$ CHO cells contain less activated Akt than do the same cells expressing $\alpha_5\beta_1$. Surprisingly, the second low Bcl-2 line, the $\alpha_5\Delta c\beta_1$, cells, activated Akt to the same degree as the $\alpha_5\beta_1$ cells. Forkhead phosphorylation closely paralleled Akt activation in the three cell lines. Thus, Akt activation upon cell attachment is induced by $\alpha_5\beta_1$, but not $\alpha_v\beta_1$, and the α_5 cytoplasmic domain is not needed for this effect.

Recruitment of the adapter protein Shc is a possible initiator of Akt activation in the $\alpha_5\beta_1$ cells. A subset of integrins that includes $\alpha_5\beta_1$ and $\alpha_v\beta_3$, but possibly not $\alpha_v\beta_1$, can recruit the adaptor protein Shc, and this event is independent of the α_5 cytoplasmic tail [Wary et al., 1996, 1998]. Shc recruits Grb2/SOS and activates Ras, which in turn activates PI3K and Akt [Khwaja et al., 1997]. That the $\alpha_5\beta_1$ and $\alpha_5\Delta c\beta_1$ integrins could activate Akt through Shc and Ras would agree with previous results showing that forced expression of Shc or activated Ras promotes Bcl-2 expression and survival in the $\alpha_v\beta_1$ cells [Matter and Ruoslahti, 2001].

One or more of the pathways from integrins to Bcl-2 expression appear to be independent of Akt and require the α_5 subunit cytoplasmic domain, as the $\alpha_5\Delta c\beta_1$cells activated Akt, but did not up-regulate Bcl-2. One of them would be

FAK pathway. FAK is involved in integrin-mediated cell survival, and forced expression of activated FAK up-regulates Bcl-2 expression in cells that lack $\alpha_5\beta_1$ [Matter and Ruoslahti, 2001]. Our results show that both the $\alpha_v\beta_1$ and $\alpha_5\Delta c\beta_1$ cells activated FAK less effectively than the $\alpha_5\beta_1$ cells. The exact mechanisms of the FAK-mediated up-regulation of Bcl-2 remain to be determined, but lack of FAK activation is likely to contribute to the low Bcl-2 induction and poor apoptosis resistance of the $\alpha_v\beta_1$ and $\alpha_5\Delta c\beta_1$ cells.

Another main pathway responsible for the low Bcl-2 expression in the $\alpha_5\Delta c\beta_1$ cells involves a CaMK, probably CaMK IV. The CaMK family includes CaMK I, CaMK IV, and CaMK kinase. The members of this family are activated upon elevation of intracellular Ca^{2+} levels [Soderling, 1999]. CaMK IV is present both in the nucleus and the cytoplasm, as is CaMK kinase, whereas CaMK I is exclusively cytosolic [Corcoran and Means, 2001]. CaMK IV regulates gene expression through the activation of several transcription factors including NF-κB and CREB [Sun et al., 1996; Corcoran and Means, 2001; Jang et al., 2001], two transcription factors capable of up-regulating Bcl-2 expression. Our laboratory recently showed that cell spreading increases the nuclear calcium concentration, and provided evidence that CaMK IV was activated [Itano et al., 2003]. We find that inhibiting CaMK IV down-regulated Bcl-2 expression and increased apoptosis of $\alpha_5\beta_1$ cells, and decreased the intracellular activities of NF-κB and CREB in $\alpha_5\beta_1$ and $\alpha_v\beta_1$ cells. Moreover, chelating intracellular (including nuclear) Ca^{2+} also down-regulated Bcl-2 expression and increased apoptosis in $\alpha_5\beta_1$ cells. These results show that CaMK IV can play a role in integrin-mediated Bcl-2 up-regulation and cell survival. As the $\alpha_5\Delta c\beta_1$ cells spread poorly on fibronectin, it seems likely that the CaMK IV pathway is defective in these cells. Taken together, these results suggest that the $\alpha_5\beta_1$ integrin supports Bcl-2 expression and cell survival through the cooperation of Akt, FAK, and CaMK IV pathways. FAK is ineffectively activated both in the $\alpha_v\beta_1$ and $\alpha_5\Delta c\beta_1$ cells, but $\alpha_v\beta_1$ specifically lacks the ability to activate Akt, and $\alpha_5\Delta c\beta_1$ fails to activate CaMK IV.

Akt and CaMK IV exert their effects on Bcl-2 expression by regulating transcription factors. Endogenous activity of NF-κB and CREB was higher in the $\alpha_5\beta_1$ and $\alpha_v\beta_1$ cells than in the $\alpha_5\Delta c\beta_1$ cells, and inhibiting either the Akt or CaMK pathway suppressed the activity of NF-κB and CREB. Moreover, transfecting either NF-κB or CREB into the cells induced Bcl-2 transcription and protein expression. Forkhead, which suppresses Bcl-2 transcription, was phosphorylated (inactivated) in $\alpha_5\beta_1$ and $\alpha_5\Delta c\beta_1$ cells, but not in the $\alpha_v\beta_1$ cells. The effects of these transcription factors on Bcl-2 levels were consistent with apoptosis levels in the cells. Thus, it seems that $\alpha_5\beta_1$ integrin induces Bcl-2 expression and cell survival by activating NF-κB and CREB, and by inactivating forkhead. The absence of Akt activation by $\alpha_v\beta_1$ allows forkhead to remain active, explaining the lack of a Bcl-2 response even in the presence of active NF-κB and CREB. The $\alpha_5\Delta c\beta_1$ integrin

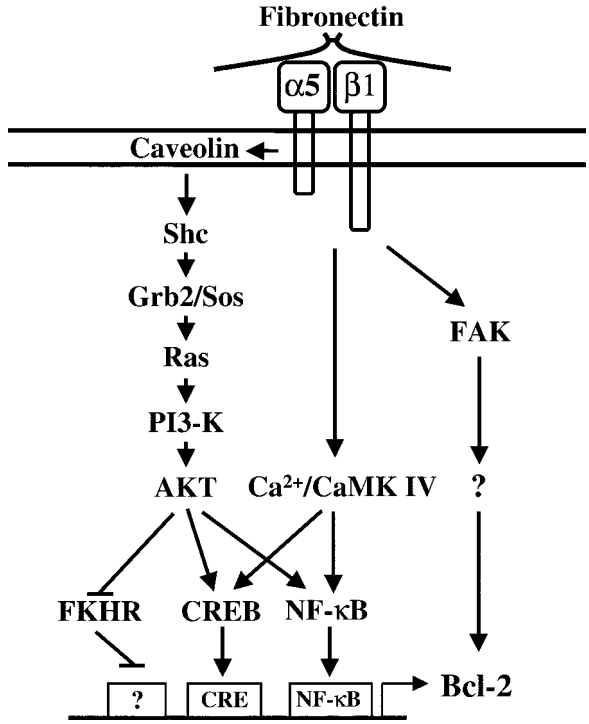

Fig. 6. Schematic diagram of the $\alpha_5\beta_1$ integrin-induced signaling pathways leading to Bcl-2 up-regulation. The $\alpha_5\beta_1$ integrin supports Bcl-2 expression and cell survival through the cooperation of Akt, FAK, and CaMK IV pathways. Akt and CaMK IV activate the positive transcription factors NF-κB and CREB, and Akt inhibits the negative transcriptional regulator forkhead. Recruitment of Shc through the caveolin is a possible initiator of Akt activation by the $\alpha_5\beta_1$ integrin. Shc is known to recruit Grb2/Sos and activate Ras, which in turn activates PI3K/Akt pathway. Cell spreading increases the nuclear calcium concentration with subsequent activation of CaMK IV. The exact mechanisms of the FAK-mediated up-regulation of Bcl-2 remain to be determined. FAK is ineffectively activated both by the $\alpha_v\beta_1$ and $\alpha_5\Delta c\beta_1$ integrins, but $\alpha_v\beta_1$ specifically lacks the ability to activate Akt, and $\alpha_5\Delta c\beta_1$ fails to activate CaMK IV.

inactivates forkhead, but does not activate NF-κB and CREB, presumably because of lack of CAMK IV activation. The α5β1 integrin-induced signaling pathways leading to Bcl-2 up-regulation are illustrated in Figure 6.

Integrin-mediated regulation of transcription factors has been shown before. For example, the $\alpha_5\beta_1$ integrin has been shown to activate NF-κB-dependent gene expression, which is important for angiogenesis [Klein et al., 2002]. The $\alpha_v\beta_3$ integrin activates NF-κB, inducing osteoprotegerin expression, which promotes endothelial cell survival [Scatena et al., 1998; Malyankar et al., 2000]. Our results show that the $\alpha_5\beta_1$ integrin inactivates forkhead transcription factors. These factors have been found to cause apoptosis by inducing the expression of cell death genes such as the Fas ligand gene [Brunet et al., 1999]. This is the first report showing that forkhead transcription factors can down-regulate the expression of anti-apoptotic Bcl-2 gene. Our results indicate that several integrin-mediated survival pathways converge on NF-κB, CREB, and forkhead. The balance of the opposing activities of transcription factors determines the level of Bcl-2 expression and regulates cell survival.

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