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Bcl-2 induces cyclin D_1 promoter activity in human breast epithelial cells independent of cell anchorage

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Abstract

Cyclin D_1 expression is co-regulated by growth factor and cell adhesion signaling. Cell adhesion to the extracellular matrix activates focal adhesion kinase (FAK), which is essential for cyclin D_1 expression. Upon the loss of cell adhesion, cyclin D_1 expression is downregulated, followed by apoptosis in normal epithelial cells. Since bcl-2 prevents apoptosis induced by the loss of cell adhesion, we hypothesized that bcl-2 induces survival signaling complementary to cell adhesion-mediated gene regulation. In the present study, we investigated the role of bcl-2 on FAK activity and cyclin D_1 expression. We found that bcl-2 overexpression induces cyclin D_1 expression in human breast epithelial cell line MCF10A independent of cell anchorage. Increased cyclin D_1 expression in stable bcl-2 transfectants is not related to bcl-2-increased G_1 duration, but results from cyclin D_1 promoter activation. Transient transfection studies confirmed anchorage-independent bcl-2 induction of cyclin D_1 promoter activity in human breast epithelial cell lines (MCF10A, BT549, and MCF-7). We provide evidence that bcl-2 induction of cyclin D_1 expression involves constitutive activation of focal adhesion kinase, regardless of cell adhesion. The present study suggests a potential oncogenic activity for bcl-2 through cyclin D_1 induction, and provides an insight into the distinct proliferation-independent pathway leading to increased cyclin D_1 expression in breast cancer. *Cell Death and Differentiation* (2001) **8,** 44–50.

Keywords: Bcl-2; cyclin D, promoter; cell anchorage

Abbreviations: FAK, focal adhesion kinase; CDKs, cyclin-dependent kinases; ECM, extracellular matrix; ER, endoplasmic reticulum; TIMP-1, tissue inhibitor of metalloproteinase-1; poly-HEMA, polyhydroxyethylmethacrylate

Introduction

The D-type cyclins are early G_1 cyclins that regulate G_1-S-phase progression. The human *cyclin D_1* gene, also known as PRAD1, D11S287 or BCL-1, is strongly implicated as an oncogene in several types of human tumors, including parathyroid adenomas. B-cell lymphomas, squamous cell carcinomas of the head, neck and esophagus, and breast cancer.[1] In a transgenic mouse model, cyclin D_1 overexpression in the mammary gland induced hyperplasia.[2,3] Cyclin D_1-deficient mice develop to term with reduced body size, symptoms of neurological impairment, and increased apoptosis in their retinas.[4,5] In adult female mice lacking *cyclin D_1*, the breast epithelial cells fail to undergo pregnancy-associated proliferation and differentiation. These studies suggest a critical role for cyclin D_1 in the regulation of cell survival and growth. In addition to its role in cell cycle regulation through activation of cyclin-dependent kinases (CDKs), CDK-independent oncogenic activity of cyclin D_1 has been reported to include estrogen receptor activation.[6,7] Cyclin D_1 binds to the hormone binding domain of the estrogen receptor and enhances its transcription factor activity.[6,7] These studies clearly suggest the significance of cyclin D_1 expression in carcinogenesis, especially in breast cancer development.

Cyclin D_1 expression is co-regulated by cell adhesion and growth factor receptor signaling.[8] Growth factors activate signal transduction pathways, including the ras-raf-p42/p44MAPK cascade and phospholipid turnover, triggering cell cycle progression from G_0 to G_1. Activation of p42/p44MAPK,[9–12] or $p60^{src}$ pathways[13] induce cyclin D_1 expression in a cell adhesion dependent fashion.[14] Integrins are heterodimeric cell surface receptors that mediate cell adhesion to the extracellular matrix (ECM) and transduce biochemical signals, including activation of focal adhesion kinase (FAK).[15–19] Neutralizing antibodies against integrins induce cell detachment followed by apoptosis in epithelial cells.[20] In the absence of cell adhesion, cyclin D_1 expression is downregulated at both RNA and protein levels, and cell cycle transition through the late G_1 restriction point is inhibited, leading to apoptotic cell death.[8,20–22] Recent studies suggest that FAK is responsible for cell adhesion-regulation of cyclin D_1 gene expression and apoptosis.[22,23] A dominant-negative FAK mutant that prevents activation of endogenous FAK downregulates cyclin D_1 expression.[23] Consistently, FAK activation increases cyclin D_1 expression[23] and rescues cells against apoptosis induced by loss of cell anchorage.[22]

Bcl-2 is a major gene product with known anti-apoptotic activity. Bcl-2 prevents cytochrome *c* release from the

mitochondria and inhibits the activation of caspases, a group of cysteine proteases that initiate the apoptotic process.[24] However, recent studies suggest pleiotropic roles for bcl-2 in the modulation of signaling molecules including Ca^{2+}, transcription factors, and signaling kinases.[21,25,26] Thus, bcl-2 regulation of apoptosis may include modulation of gene expression critical for cell survival. Since bcl-2 inhibits apoptosis induced by loss of cell anchorage,[20–22] we hypothesized that bcl-2 modulates cell adhesion-mediated gene expression. In this study, we examined the role of bcl-2 in regulating FAK activity and cyclin D_1 expression in human breast epithelial cells in the presence and absence of cell adhesion.

Results

Bcl-2 upregulates cyclin D_1 expression and increases the G_1 duration of human breast epithelial cells

Immunoblot analysis of the cyclin D_1 protein was performed to examine whether bcl-2 modulates cyclin D_1 expression. As shown in Figure 1, bcl-2 overexpressing MCF10A clones exhibited a 3–4-fold increase of cyclin D_1 protein compared to control MCF10A cells transfected with a neomycin resistant vector (neo, >50 clones were pooled together). The fold of induction was estimated after normalizing the intensity of bcl-2 and cyclin D_1 bands to the β-actin band.

Cyclin D_1 expression is known to be regulated during the cell cycle: in early G_1, cyclin D_1 expression is induced by extracellular mitogens, and its RNA and protein levels remain high during the G_1 phase.[28] Since recent studies showed that bcl-2 increases the length of the G_1 phase in mouse lymphoid cells,[29,30] we asked whether increased cyclin D_1 protein levels were a reflection of an increased G_1 population in bcl-2 overexpressing MCF10A cells. To this end, we determined what fractions of control and bcl-2 overexpressing cells were in the G_1 phase. Flow cytometric analysis revealed that ~28% of logarithmically growing control cells were in the G_1 phase, while ~32% of bcl-2 overexpressing cells were in the G_1 phase. This small difference in the G_1 population is not likely to be responsible for a 3–4-fold difference in cyclin D_1 expression between the control and bcl-2 overexpressing MCF10A cells. To further investigate whether bcl-2 induction of cyclin D_1 is related to cell cycle regulation or not, we first examined whether bcl-2 modulates G_1 duration in MCF10A cells. Control and bcl-2 overexpressing MCF10A cells were serum-starved for 48 h, before the cell cycle was induced by serum and growth factor treatment. The bcl-2 effect on the length of the G_1 phase was examined by flow cytometric analysis. The percentage of G_0/G_1 population was comparable among serum-starved control and bcl-2 overexpressing MCF10A cells (~80% were in G_0/G_1). About 10 h after serum treatment, cell cycle progression into the S-phase was noticed in control vector transfected cells. As shown in Figure 2, a twofold increase in S population required approximately 15 h in the control cells following serum-treatment, whereas 17–21 h were required in the bcl-2 overexpressing MCF10A clones. This showed that bcl-2 prolongs G_1 duration in human breast epithelial cells as in mouse lymphoid cells.[29,30] We next examined the cyclin D_1 levels in cells entering the cell cycle in a synchronized manner. If the increased cyclin D_1 expression was due to a larger G_1 population resulting from a longer G_1 phase in asynchronously growing cells overexpressing bcl-2, cyclin D_1 levels should be comparable between the control and bcl-2 overexpressing cells when the cell cycle is synchronized. As shown in Figure 3A, cyclin D_1 expression was higher in bcl-2 overexpressing cells than the control cells at all time points from 0–20 h. This suggested that bcl-2-increased cyclin D_1 gene expression is not directly related to cell cycle distribution. It should be noted that bcl-2 expression was serum inducible and that the bcl-2 expression levels in the bcl-2 transfected cells remained higher than those of control cells even in quiescence (Figure 3B). To assess the functional significance of cyclin D_1 induction in bcl-2 overexpressing cells, we measured cyclin D_1-associated kinase activity *in vitro* using purified GST-pRb fusion protein as a substrate. As shown in Figure 3C, bcl-2 induction of cyclin D_1 expression resulted in enhanced cyclin D_1-associated kinase activity.

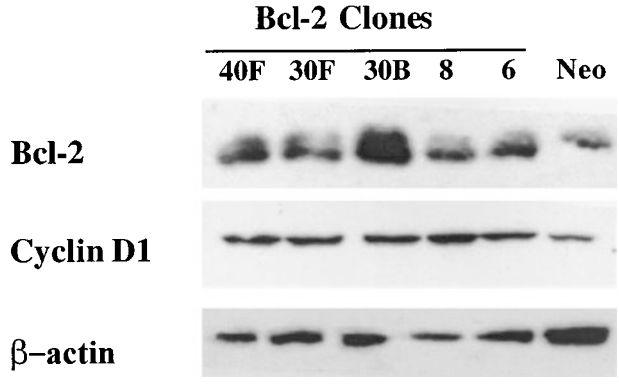

Figure 1 Bcl-2 induces cyclin D_1 protein expression in MCF10A cells. Lysates (25 μg/lane) of bcl-2-overexpressing MCF10A clones (40F, 30F, 30B, 8 and 6) and vector-transfected clones (Neo, >50 clones were pooled together) were subjected to immunoblot analysis with an anti-bcl-2, anti-cyclin D_1 or anti-β-actin antibody. Detection of the antigen was performed using ECL

Bcl-2 activates cyclin D_1 promoter activity

To determine whether bcl-2 overexpression results in upregulation of cyclin D_1 expression at the transcriptional level, we examined cyclin D_1 promoter activity in control (neo) or bcl-2 overexpressing MCF10A cells. To this end, a luciferase reporter gene under the control of 964 bp cyclin D_1 promoter (−964CDLUC) was introduced into the control or bcl-2 overexpressing MCF10A cells. As shown in Figure 4A, cyclin D_1 promoter activity was fivefold higher in bcl-2 overexpressing cells.

To exclude the possibility that increased cyclin D_1 promoter activity in bcl-2 overexpressing MCF10A cells results from clonal selection and/or long term culture with stable bcl-2 expression, we examined the bcl-2 role in

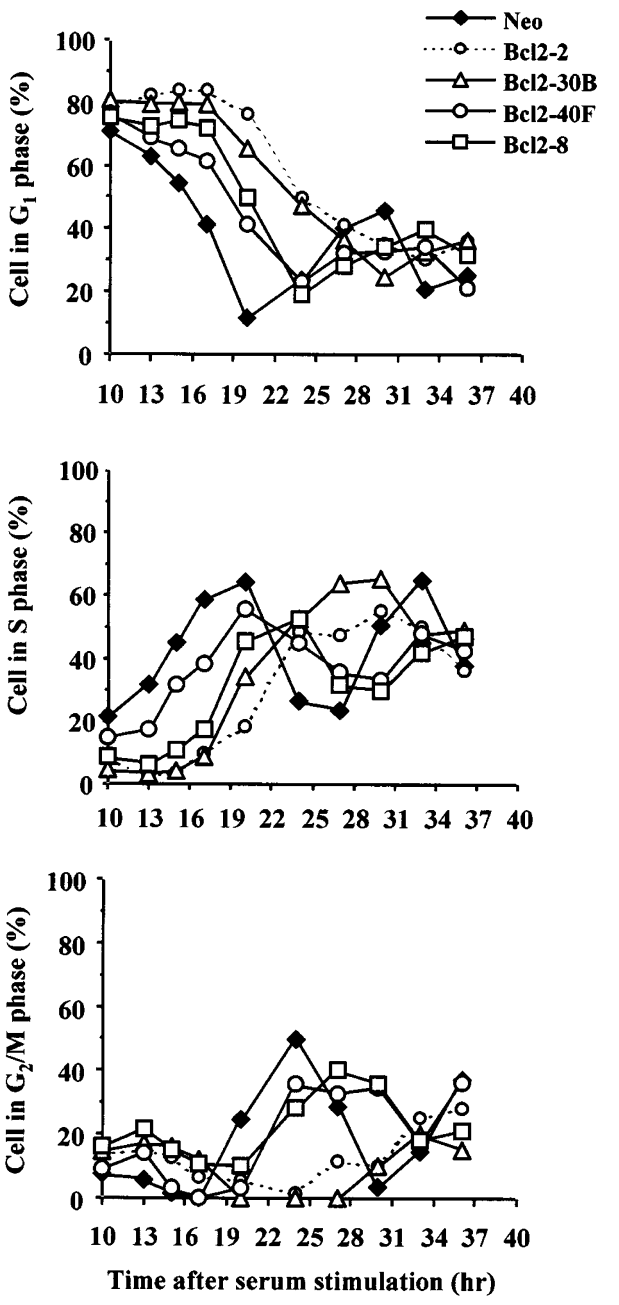

Figure 2 Bcl-2 increases G_1 duration in MCF10A cells. The kinetics of serum-induced cell cycle progression were determined by flow cytometric analysis. The proportions of cells in each cell cycle phase were plotted at the time indicated (between 10 and 40 h) after serum treatment. Vector-transfected (Neo, pooled population) or bcl-2-overexpressing MCF10A clones (2, 30B, 40F and 8) were analyzed

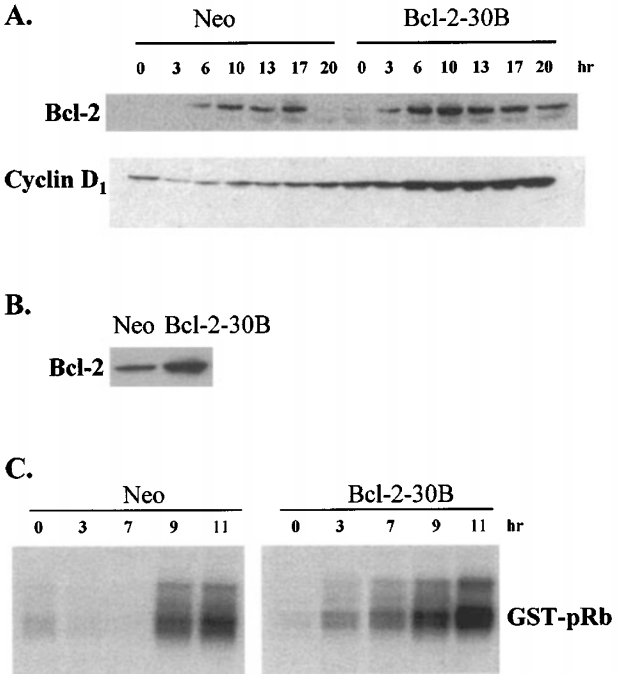

Figure 3 Bcl-2-induced cyclin D_1 expression is not related to bcl-2-increased G_1 duration. (**A**) Lysates (25 μg/lane) of vector transfected (Neo) or MCF10A bcl-2-30B cells prepared at the indicated time points (shown above) during serum-induced cell cycle progression were subjected to immunoblot analysis with an anti-bcl-2 or anti-cyclin D_1 antibody. Detection of the antigen was performed using ECL. (**B**) Lysates (100 μg/lane) of vector transfected (Neo) or MCF10A bcl-2-30B cells cultured in serum-free medium for 48 h were subjected to immunoblot analysis with an anti-bcl-2 antibody. Detection of the antigen was performed using ECL. (**C**) Cyclin D_1 complex was immunoprecipitated from the cell extracts of vector transfected (Neo) or MCF10A bcl-2-30B cells prepared at the indicated time points (shown above) during serum-induced cell cycle progression. Kinase activity of the complexes was measured in the presence of [γ-^{32}P]ATP using purified GST-pRb fusion protein as a substrate

cyclin D_1 promoter activation by transient transfection. As shown in Figure 5A, cyclin D_1 promoter activity in MCF10A cells was significantly induced by transient transfection with bcl-2 expression plasmid. As a control experiment, the effects of bcl-2 expression on luciferase reporter gene expression under SV40 or cyclin E promoter were also examined. There was no increase in luciferase activity in either SV40 or cyclin E promoter when co-transfected with bcl-2 expression plasmid (data not shown), suggesting cyclin D_1 promoter activation was bcl-2 specific.

We next examined whether bcl-2 induction of cyclin D_1 promoter activity is confined to MCF10A cells, or if it has a general effect in human breast epithelial cells. To this end, we examined the bcl-2 effect on cyclin D_1 promoter activity in BT549 and MCF-7 cells, two human breast carcinoma cell lines. Cyclin D_1 promoter activity was enhanced by bcl-2 in both cell lines. By transient transfection of 1 μg bcl-2 expression plasmid, Cyclin D_1 promoter activity was enhanced more than fourfold in BT549 cells, and more than 10-fold in MCF-7 cells (Figure 5B,C).

Bcl-2 activation of cyclin D_1 promoter activity is independent of cell adhesion

The cyclin D_1 promoter activity was reduced 75% in suspension culture (using polyMEHA-coated dishes which prevents cell adhesion) compared to the monolayer culture of control MCF10A cells (Figure 4A). The cyclin D_1 protein levels also decreased (approximately 50%) in suspension culture (Figure 4B). This is in agreement with previous reports that growth factor-mediated cell proliferation signals are insuffi-

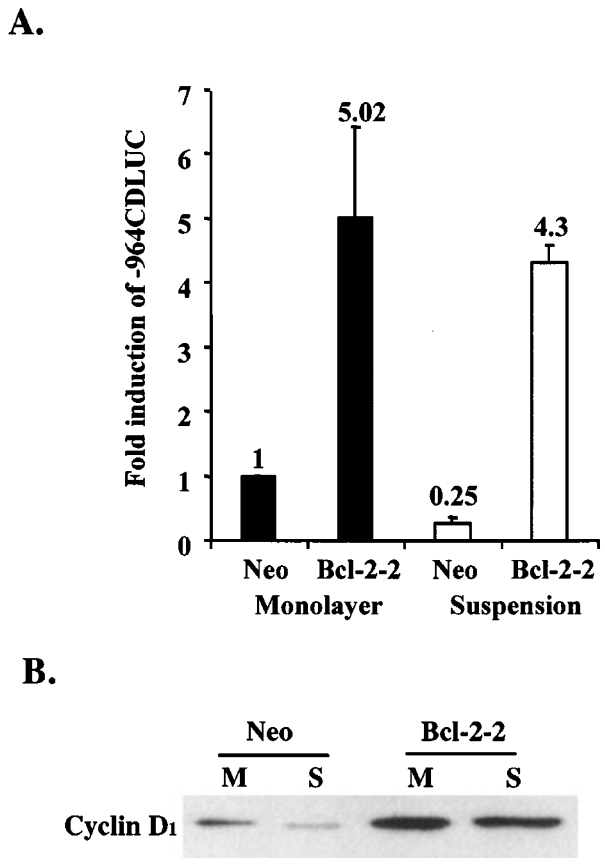

Figure 4 Cyclin D_1 promoter activity is enhanced in bcl-2 overexpressing MCF10A cells regardless of cell anchorage. (**A**) Control (Neo) or bcl-2 overexpressing MCF10A cells (Bcl-2) were co-transfected with 3 μg of luciferase reporter construct (−964CDLUC) and 0.15 μg of the β-galactosidase expression plasmid (pMDV-lacZ) using FuGENE 6 reagent. Cells were cultured on regular plates (monolayer) or polyHEMA coated dishes (suspension) for 24 h. The luciferase activity in 1 μg of cell lysate was normalized to β-galactosidase activity. The luciferase activity in the control cells grown on monolayer was arbitrarily given as 1. The data represent the average of six separate transfection experiments. Error bars represent S.D. of the mean. (**B**) Lysates (25 μg/lane) of control (Neo) or bcl-2 overexpressing MCF10A cells (Bcl-2) cells grown in monolayer (M) or suspension (S) were subjected to immunoblot analysis with an anti-cyclin D_1 antibody. Detection of the antigen was performed using ECL

cient for cyclin D_1 expression and cell adhesion is required for its expression.[8,14] In contrast, cyclin D_1 promoter activity remained high in bcl-2 overexpressing MCF10A cells regardless of cell anchorage. Cyclin D_1 protein expression was also stable in bcl-2 overexpressing MCF10A cells in the absence of cell adhesion (Figure 4B). This study showed that bcl-2 induction of cyclin D_1 promoter activity is independent of cell adhesion. Interestingly, bcl-2 upregulation of cyclin D_1 promoter activity was more drastic in the absence of cell anchorage: bcl-2 resulted in a ~17-fold increase in cyclin D_1 promoter activity in suspension culture of MCF10A cells, compared with a ~fivefold induction in monolayer culture (Figure 4A).

We further investigated anchorage-independent bcl-2 induction of cyclin D_1 promoter activity in human breast epithelial cells by transient transfection studies. Transient transfection of bcl-2 expression plasmid resulted in increased cyclin D_1 promoter activity independent of cell adhesion in all three human breast epithelial cell lines tested (Table 1).

Bcl-2 activates FAK regardless of cell anchorage

Since increasing evidence indicates that FAK activity is critical for anchorage-independent cell survival and cyclin D_1 expression,[22,23,31] we examined whether bcl-2-mediated cyclin D_1 induction involves increased expression levels and/or activation of FAK. The expression levels of FAK were not altered by bcl-2 overexpression, as determined by immunoblot analysis using an anti-FAK mAb (Figure 6A). We next examined whether bcl-2 modulates FAK activity. To this end, the FAK protein was immunoprecipitated with an anti-FAK mAb and the active form was detected by immunoblot analysis using an anti-phosphotyrosine antibody. As shown in Figure 6B, FAK is more efficiently activated in bcl-2 overexpressing cells than in the control cells. Since studies showed that FAK activation requires cell anchorage,[18,22] we asked whether bcl-2 upregulation of FAK activation also required cell anchorage. To this end, we cultured control and bcl-2 overexpressiing cells in suspension for 12 h and examined tyrosine-phosphorylated FAK. As shown in Figure 6C, bcl-2 constitutively activated FAK regardless of cell anchorage. This suggests that bcl-2 upregulates cyclin D_1 expression through constitutive activation of cell adhesion signaling pathways independent of cell adhesion.

Discussion

The present study showed that bcl-2 upregulates cyclin D_1 expression in human breast epithelial cells. Effects of increased cyclin D_1 expression on G_1/S transition appear to be cell type-specific. While cyclin D_1 shortens the G_1 phase in rat embryonic fibroblasts, cyclin D_1 overexpression results in an increased fraction of cells in the G_1 phase in mouse mammary epithelial cells.[32,33] In MCF10A cells, increased expression of cyclin D_1 is associated with increased G_1 duration, the same as in mouse mammary epithelial cells. This suggests that cyclin D_1 may be unrelated to cell cycle acceleration in breast epithelial cells. Consistently, cyclin D_1 overexpression in invasive ductal type or lobular carcinoma of the breast was not associated with Ki-67 expression, a protein present in all dividing cells.[34]

Bcl-2 is often overexpressed in human cancer cells including breast epithelial cells, and its oncogenic activity was thought to result from inhibition of apoptosis, thereby increasing cell number.[35–37] In this study, we showed that bcl-2 induces expression of cyclin D_1, an oncogene involved in breast cancer development.[2,3,6,7] This suggests a potential bcl-2 oncogenic activity in breast cancer through cyclin D_1 induction. At present, it is unclear whether cyclin D_1 is involved in bcl-2 apoptosis regulation. Growth and survival of normal fibroblasts and epithelial cells require cell adhesion. Following the loss of cell adhesion, cyclin D_1 expression decreases and cyclin E activity is inhibited, while the level of cyclin E expression remains the same.[8]

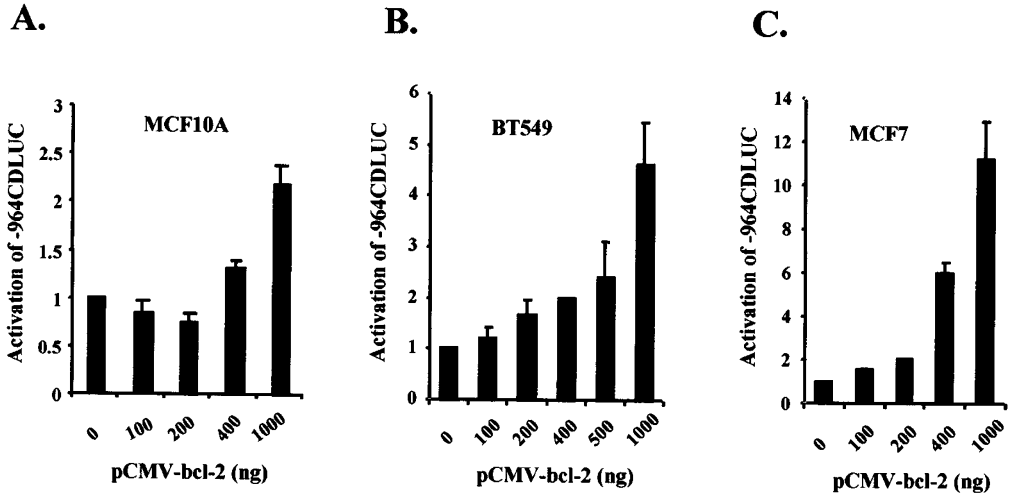

Figure 5 Bcl-2 induces cyclin D_1 promoter activity in MCF10A, BT549 and MCF-7 cells. Three μg of -964CDLUC plasmid, 0.15 μg of pMDV-lacZ and increasing amounts of bcl-2 expression vector were co-transfected into MCF10A **(A)** BT549 **(B)** or MCF7 **(C)** cells. The plasmid pCRII (purchased from Stratagene) was supplemented to use the same amount of DNA in each transfection. Cell lysate was harvested 30 h after transfection and the luciferase activity in 1 μg of cell lysate was normalized to β-galactosidase activity to control the transfection efficiencies. The luciferase activity in cells transfected without bcl-2 expression plasmid was arbitrarily given as 1. Error bars represent S.D. of the mean triplicate samples

Table 1 Bcl-2 activation of cyclin D_1 promoter activity in suspension culture

	pCMV-neo	pCMV-bcl-2
BT549	1	2.93 ± 1.67
MCF7	1	5.06 ± 1.34
MCF10A	1	3.78 ± 0.10

BT549, MCF-7, and MCF10A cells were transiently transfected for 24 h with 3 μg of -964 CDLUC, 0.15 μg of pMDV-lacZ, and 0.5 μg of bcl-2 expression vector (pCMV-bcl-2) or control vector (pCMV-neo). Cells were trypsinized and cultured in suspension for an additional 24 h before luciferase activity assay. The luciferase activity in cells co-transfected with pCMV-neo was arbitrarily given as 1. Luciferase activity with the pCMV-bcl-2 expression plasmid was normalized to luciferase activity with pCMV-neo in each cell line. Data represents the average $\pm$ S.D. of eight independent transfection experiments

These cells undergo apoptosis or cell cycle arrest. Bcl-2 was shown to prevent apoptotic cell death induced by the loss of cell adhesion. Interestingly, ectopic expression of cyclin D_1, but not cyclin E, was shown to induce anchorage-independent cell cycle progression,[31] suggesting a role for cyclin D_1 in cell-adhesion mediated cell cycle regulation. Taken together, cyclin D_1 may be involved in specified type of apoptosis regulated by bcl-2 (such as apoptosis induced by loss of cell adhesion).

Bcl-2 is localized mainly to the membranes of the mitochondria and the endoplasmic reticulum (ER),[38] which represent the main intracellular storage sites for Ca^{2+}. Bcl-2 was shown to regulate Ca^{2+} homeostasis in the mitochondria as well as in ER.[25,39] Bcl-2 regulation of intracellular Ca^{2+} may be critical for FAK activity as previously suggested.[40] Alternatively, bcl-2-mediated changes in signaling molecules such as Ca^{2+} may modulate gene expression of the adhesion related genes, which in turn regulate FAK activity. Consistent with this, we recently reported that bcl-2 induces expression of tissue inhibitor of metalloproteinase-1 (TIMP-1), and TIMP-1 overexpression leads to constitutive activation of FAK.[41]

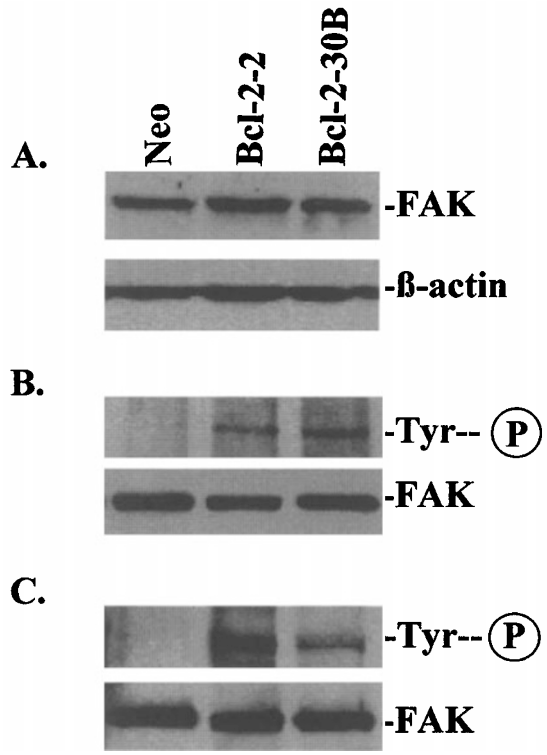

Figure 6 Bcl-2 constitutively activates focal adhesion kinase regardless of cell adhesion. (**A**) Lysates (50 μg/lane) of control (Neo or bcl-2 overexpressing MCF10A clone-2 and -30B were subjected to immunoblot analysis using an anti-FAK mAb and detection by ECL. To control the amount of proteins loaded in each lane, the identical blot was probed with a β-actin mAb. (**B**, **C**) Cells were cultured (12 h) in monolayer (**B**) or in suspension (**C**) and solubilized in lysis buffer. The lysates (400 μg) were then immunoprecipitated with an anti-FAK mAb and protein G Sepharose beads. The immunoprecipitates were resolved by reducing SDS–PAGE followed by immunoblot analysis using an anti-phosphotyrosine mAb (top panels). To confirm the amount of immunoprecipitated FAK protein in each sample, the identical blot was probed with the anti-FAK mAb (bottom panels)

Although the exact mechanism of bcl-2 activation of FAK and cyclin D_1 promoter is not understood, this study provides an insight into the distinct proliferation-independent signaling pathway for cyclin D_1 induction by bcl-2 in human breast epithelial cells.

Materials and Methods

Monolayer cell culture

Immortalized nonmalignant human breast epithelial MCF10A cells were obtained from the Barbara Ann Karmanos Cancer Institute (KCI), Detroit, MI, USA. MCF10A cells and the stable bcl-2 transfectants were cultured as previously described.[27] The human breast carcinoma cell line BT549 was obtained from Dr. EW Thompson and maintained in RPMI1640 medium with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). The human breast carcinoma cell line MCF-7 was cultured in DMEM/F12 medium with 10% donor calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Suspension culture

PolyHEMA (polyhydroxyethylmethacrylate, purchased from Aldrich Chemical Co., WI, USA) was solubilized in methanol (50 mg/ml) and diluted in ethanol to a final concentration of 10 mg/ml. To prepare polyHEMA-coated dishes, 4 ml of polyHEMA solution was placed in 100-mm petri dishes and dried in a tissue culture hood. The polyHEMA coating was repeated twice, followed by three washes with PBS. Cells were cultured in suspension using polyHEMA-coated 100-mm dishes in a 95% air and 5% CO_2 incubator.

Immunoblot analysis

Cells were lysed in SDS lysis buffer (2% SDS, 125 mM Tris-HCl, pH 6.8, 20% Glycerol). The lysates were boiled for 5 min and then clarified by a 20-min centrifugation at 4°C. Protein concentration was measured using BCA protein assay reagent (Pierce, IL, USA). Equal amounts (25 μg/lane) of protein samples in SDS sample buffer (1% SDS, 62.5 mM Tris-HCl, pH 6.8, 10% Glycerol, 5% β-mercaptoethanol, 0.05% Bromophenol Blue) were boiled for 5 min and subjected to reducing SDS–PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane. The blot was then probed with anti-bcl-2 (DAKO), anti-cyclin D_1 (CC11, AB2, Calbiochem) or anti β-actin (Sigma) antibodies. After three washes with T-TBS, the blot was incubated with the appropriate HRP-conjugated secondary antibodies. The antigen was detected using the ECL detection system according to the manufacturer's instruction (Pierce, Rockford, IL, USA).

Flow cytometric analysis

Cells were synchronized at G_0 by culturing in serum-free DMEM medium without supplementing growth factors for 48 h. Complete MCF10A medium was added, and cells were harvested at the indicated time points. Cells were fixed in 70% ethanol for 24 h at 4°C. The DNA content was measured by staining the nuclei with Hoechst 33258 at a concentration of 3 μg/ml for 15 min and counted in a fluorescence-activated cell sorter (Beckton Dickinson Immunocytometry System). Twenty thousand cells were analyzed for each time point. The percentage of cells in each phase of the cell cycle was determined by ModFit software.

Cyclin D_1-associated kinase assay

Extracts were prepared by lysing the cells in ice-cold buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 25 mM NaF, 1 mM sodium vanadate, 1 mM EDTA, 2 mM benzamidine, 1 mM PMSF, 0.5% Nonidet P-40, and 10 μg/ml leupeptin and aprotinin. After removing the precipitate by centrifugation at 14 000 r.p.m. for 15 min at 4°C, the cyclin D_1 complex was collected using the anti cyclin D_1 antibody and protein G-Sepharose. The complex was washed three times with 1.0 ml of washing buffer (1% deoxycholate, 0.5% tween 20, 50 mM Tris-HCl, pH 7.5) and once with 1 ml of 50 mM Tris (pH 7.5) and 10 mM $MgCl_2$. Kinase activity of the cyclin D_1 immune complex was measured using 2.5 μM GST-pRb protein (purchased from Santa Cruz Biotech., CA, USA) as a substrate in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM NaF, 0.1 mM sodium orthovandate, 5 μg/ml leupeptin, and [γ-^{32}P]ATP (0.01–0.3 nCi/pmol). After kinase reaction at 30°C for 30 min, samples were mixed with 2 × SDS buffer and subjected to SDS–PAGE analysis. γ-^{32}P-ATP-labeled Rb proteins were visualized by autoradiography.

Plasmids and transient transfection

The luciferase reporter construct of human cyclin D_1 promoter (−964CD1LUC) was previously described.[11,12] The human bcl-2 expression vector with the cytomegalovirus (CMV) promoter was obtained from Dr. SJ Korsmeyer (Harvard University, Boston, MA, USA). Cells were seeded into 6-well plates at 30% confluency 1 day before transfection. Transfection was carried out using FuGENE 6 reagent (Boehringer Mannheim) according to the manufacturer's protocol. Briefly, each well was transfected with 3 μg of cyclin D_1 reporter −964CD1LUC, 0.15 μg of the β-galactosidase expression plasmid pMDV-lacZ, and increasing amounts of bcl-2 expression vector. The pCRII plasmid DNA (Stratagene) was used to supplement the amount of DNA in each transfection. At 24–36 h after transfection, cells were washed with cold phosphate-buffered saline (PBS) and lysed in 300 μl reporter lysis buffer (Promega).

To examine the cyclin D_1 promoter activity in the presence or absence of cell adhesion, cells were washed with PBS and trypsinized 24 h after transfection. One-half of the cells were cultured in regular plates and the other half were cultured using polyHEMA-coated plates. Cells were cultured for an additional 24 h and harvested for analysis.

Luciferase/β-galactosidase activity assay

Luciferase and β-galactosidase activity were assayed using Luciferase assay kit (Promega) and Galacto-Light kit (Tropix, Ins.) and measured with a luminometer (Promega, Turner TD-20e). The Luciferase activity was normalized to β-galactosidase activity and total protein determined by BCA protein assay kit (Pierce).

Immunoprecipitation of FAK

Cells were lysed in RIPA buffer (100 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40 and 1% SDS) containing freshly added protease inhibitors (100 μg/ml PMSF in isopropanol, 45 μg/ml aprotinin, and 1 mM Sodium Orthovanadate). The lysates were centrifuged for 15 min at 12 000 *g* to remove debris, and immunoprecipitated using an anti-FAK monoclonal antibody (Transduction Laboratories), and protein G agarose beads (Boehringer Mannheim, IN, USA). Immunoprecipitates were washed five times with RIPA buffer and resolved by reducing SDS–PAGE. Tyrosine-phosphorylated FAK proteins were detected by immunoblotting using an anti-phosphotyrosine antibody (Transduction Laboratories).

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