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Cyclin D_1 expression is co-regulated by growth factor signaling and by cell adhesion signaling. Integrins mediate cell adhesion to the extracellular matrix and transduce biochemical signals, including activation of focal adhesion kinase. Upon the loss of cell adhesion, cyclin D_1 expression is downregulated, and 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. We showed that bcl-2 overexpression induces cyclin D_1 expression in MCF10A cells. Transient transfection studies confirmed bcl-2 induction of cyclin D_1 promoter activity in MCF10A, BT549, and MCF-7 cells in an anchorage-independent manner. We provide evidence that bcl-2 induction of cyclin D_1 involves constitutive activation of focal adhesion kinase. The present study suggests a novel bcl-2 oncogenic activity through cyclin D_1 induction, and also provides an insight into the distinct proliferation-independent pathway leading to increased cyclin D_1 expression in breast cancer.

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(4) TABLE OF CONTENTS

(5) Introduction

The bcl-2 family consists of gene products that either promote or inhibit apoptosis ((1)). One of the critical functions of the bcl-2 family members is to regulate the activation of caspases, a group of cysteine proteases that cleave substrates after aspartic acid residues and initiate the apoptotic processes ((2)). Caspases exist as proenzymes in the cytosol and become activated through proteolysis following apoptotic signaling ((2)). Cytochrome C and CED-4 family members such as Apaf-1 appear to be critical for caspase activation ((3)). The bcl-2 gene product at the outer mitochondrial membrane prevents cytochrome C release and inhibits CED-4 activation of caspases ((1,4)). However, increasing evidence suggests pleiotropic roles for bcl-2 in apoptosis regulation ((5-7)). In addition to the mitochondrial membrane, bcl-2 is also localized to the outer nuclear membrane and endoplasmic reticulum (ER). We have previously shown that bcl-2 increases calcium uptake through upregulation of endoplasmic reticulum calcium pump (SERCA) expression, enhancing the lumenal Ca2+ concentration of ER in MCF10A cells ((5)). A recent report showed that bcl-2 activates the transcription factor NFkB through degradation of its inhibitor IkBa ((7)). Bcl-2 was also shown to inhibit activation of Jun-N-Terminal Kinases (JNKs) known to play a role in apoptosis ((6)). This suggests that bcl-2 inhibition of apoptosis may involve regulation of gene expression through modulation of central signaling molecules including Ca2+ loads, transcription factors and kinase activity. We have investigated the roles of bcl-2 in the regulation of gene expression that are critical for apoptosis regulation and oncogenic activity.

(6) Body of Report

The *cyclin D1* gene is amplified in approximately 20% of mammary carcinomas, and the protein is overexpressed in approximately 50% of breast diseases ((8) (9-12)). The transition of hyperplasia to carcinoma appears to be associated with increased cyclin D_1 mRNA expression. Cyclin D_1 expression is co-regulated by integrin and growth factor receptor signals ((13)). Growth factors activate signal transduction pathways, including the ras-raf-p42/p44MAPK cascade and phospholipid turnover, and trigger cell cycle progression from G_0 to G_1. Activation of p42/p44MAPK ((14,15)) or $p60^{src}$ pathways ((16)) induce cyclin D_1 expression in a cell adhesion dependent fashion ((17)). 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) ((18-21)). Neutralizing antibodies against integrins induce cell detachment followed by apoptosis in epithelial cells ((22)). 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 ((13,22)). A recent study suggests a critical role for FAK in cyclin D_1 gene expression. A dominant-negative FAK mutant that competes with endogenous FAK in focal contacts downregulates cyclin D_1 expression. Consistently, wild-type FAK increases cyclin D_1 expression ((23)).

We have previsouly reported that bcl-2 induces cyclin D1 promoter acitvity in MCF10A cells. During the 1998-1999 funding period, we examined the role of bcl-2 in

regulating cyclin D1 expression in human breast epithelilal cell lines (MCF10A, MCF07 and BT549) in the presence and absence of cell adhesion.

Methods

Monolayer cell culture. Immortalized nonmalignant human breast epithelial MCF10A cells were obtained from the Barbara Ann Karmanos Cancer Institute (KCI), Detroit, MI. MCF10A cells and the stable bcl-2 transfectants were cultured as previously described (Upadhyay et al., 1995). The human breast carcinoma cell line BT549 was obtained from Dr. E. W. 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 and penicillin (100 units/ml) and streptomycin (100 μg/ml).

Suspension culture. PolyHEMA (polyhydroxyethylmethacrylate, purchased from Aldrich Chemical Co., WI) 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 onto 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, 125mM Tris-HCl, pH 6.8, 20% Glycerol). The lysates were boiled for 5 min and then clarified by a 20-minute centrifugation at 4°C. Protein concentration was measured using BCA protein assay reagent (Pierce, IL). Equal amounts (25 μg/lane) of protein samples in SDS sample buffer (1% SDS, 62.5mM Tris-HCl, pH 6.8, 10% Glycerol, 5% β-mercaptoethanol, 0.05% Bromophenol Blue) were boiled for 5 minutes 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), anti-$p21^{WAF1/CIP1}$ (05-345, Upstate Biotechnology), 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 (Pierce, Rockford, IL) according to the manufacturer's instruction.

Plasmids and transient transfection. The reporter construct of human cyclin D_1 promoter -964CD1LUC was previously described (Watanabe et al., 1996a; Watanabe et al., 1996b). The human bcl-2 expression vector with the cytomegalovirus (CMV) promoter was obtained from Dr. S. J. Korsmeyer (Harvard University, Boston, MA). Cells were seeded into 6-well plates at 30% confluency one day before transfection. Transfection was carried out by FuGENE 6 reagent (Boehringer Mannhem) according to the manufacturer's protocol. Briefly, each well was transfected with 3 μg of cyclin D_1 reporter, increasing amounts of bcl-2 expression vector, and pCRII (Stratagene) which was used to supplement the amount of DNA in each transfection. The transfection efficiency was normalized by co-transfection with 0.15 μg of the β-galactosidase expression plasmid pMDV-lacZ. Cells were washed with cold phosphate-buffered saline (PBS) and lysed in 300 μl reporter lysis buffer (Promega) 24-36 hours after transfection. Each transfection was repeated at least 6 times with two different plasmid preparations.

For the loss of cell-adhesion experiments, cells were washed with PBS and trypsinized 24 hours 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 hours and harvested for analysis. The data represent at least three independent experiments and standard errors are within 5-15% of the mean.

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 minutes at 12,000g to remove debris, and immunoprecipitated using an anti-FAK monoclonal antibody (Transduction Laboratories), and protein G agarose beads (Boehringer Mannheim, IN). Immunoprecipitates were washed 5 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).

Results

Bcl-2 activates cyclin D_1 promoter activity.

Since mounting evidence suggests the significance of cyclin D_1 expression in breast cancer, we further investigated bcl-2 regulation of cyclin D_1 expression in the present study. To examine whether bcl-2 regulates cyclin D_1 promoter activity in MCF10A cells, luciferase reporter gene under the control of 964 bp cyclin D_1 promoter was introduced into the control or bcl-2 overexpressing MCF10A cells. As shown in Fig. 4A, cyclin D_1 promoter activity was ~5- fold 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 for cyclin D_1 promoter activity in transiently transfected cells. As shown in Fig. 5A, cyclin D_1 promoter activity in MCF10A cells was significantly induced with increased amount of transiently transfected bcl-2 expression plasmid.

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 activities were enhanced by bcl-2 in both cell lines. By transient transfection of 1 μg/ml bcl-2 expression plasmid, Cyclin D_1 promoter activity was enhanced more than 4-fold in BT549 cells, and more than 10-fold in MCF-7 cells (Fig. 5B and C).

Bcl-2 activation of cyclin D1 promoter activity is independent of cell adhesion.

Cyclin D_1 promoter activity was reduced 75% in suspension culture compared to the monolayer culture of control MCF10A cells (Fig.4A). The cyclin D_1 protein levels also decreased (approximately 50%) in suspension culture (Fig. 4B). This was in agreement with the previous reports that cyclin D_1 expression requires cell adhesion, and growth factor-mediated cell proliferation signals are insufficient for its expression (Zhu et al., 1996; Le Gall et al., 1998). In contrast, cyclin D_1 promoter activity remained high in bcl-2 overexpressing MCF10A cells regardless of cell anchorage. The cyclin D_1 protein expression was also stable in bcl-2 overexpressing in the absence of cell adhesion (Fig. 4B). This study showed that bcl-2 induction of cyclin D_1 promoter activity is 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 ~5-fold induction in monolayer culture (Fig.4A).

We further investigated anchorage-independent bcl-2 induction of cyclin D_1 promoter activity in human breast epithelial cells by transient transfection studies. While cyclin D_1 promoter activity was significantly reduced in suspension culture of MCF10A cells (spontaneously immortalized "normal" breast epithelial cell line), cyclin D_1 promoter activity was comparable between suspension and monolayer cultures of BT549 and MCF7 (breast carcinoma cell lines). (Table 1). This suggests that these carcinoma cells may have acquired genetic changes that complement cell adhesion signaling. As summarized in the Table 1, 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.

Bcl-2 activates FAK regardless of cell anchorage.

Increasing evidence indicates that FAK activity is critical for anchorage-independent cell cycle transition, cell survival, and cyclin D_1 expression (Frisch et al., 1996a; Zhao et al., 1998; Resnitzky, 1997). Therefore, we examined whether bcl-2-mediated cyclin D_1 induction involves increase in expression level 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 (Fig. 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 Fig. 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 (Guan et al., 1992; Frisch et al., 1996a), we asked whether bcl-2 upregulation of FAK activation also required cell anchorage. To this end, we cultured control and bcl-2 overexpressing cells in suspension for 12 hours and examined tyrosine-phosphorylated FAK. As shown in Fig. 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.

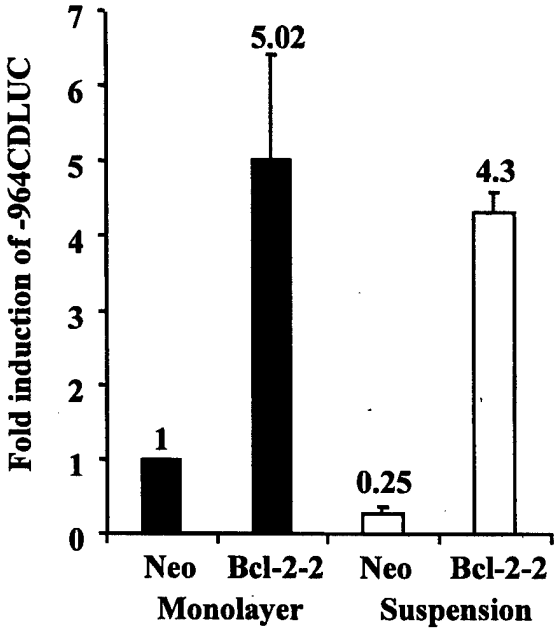
(7) Key Research Accomplishments

Bcl-2 regulation of cyclin D1 promoter activity independent of cell adhesion.

(8) Reportable Outcomes

Lin, H-M., Lee, Y. J., Li, G., Pestell, R. G., and **Kim, H.-R. C**. Bcl-2 induces cyclin D1 promoter activity in human breast epithelial cells independent of cell anchorage. Submitted for publication.

A.



B.

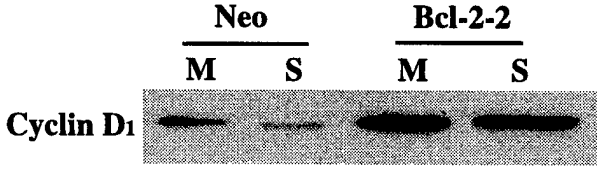


Fig. 1. Cyclin D_1 promoter activity is enhanced in bcl-2 overexpressing MCF10A cells regardless of cell anchorage.

(**A**) Vector transfected (neo) or bcl-2 overexpressing MCF10A clone 2 cells were co-transfected with 3 μg of luciferase reporter construct (–964CDLUC) and 0.15 ug 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 hours. The luciferase activity in 1μ g 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 6 separate transfection experiments. (**B**) Lysates (25 μg/lane) of vector transfected (Neo) or bcl-2 overexpressing MCF10A clone 2 (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.

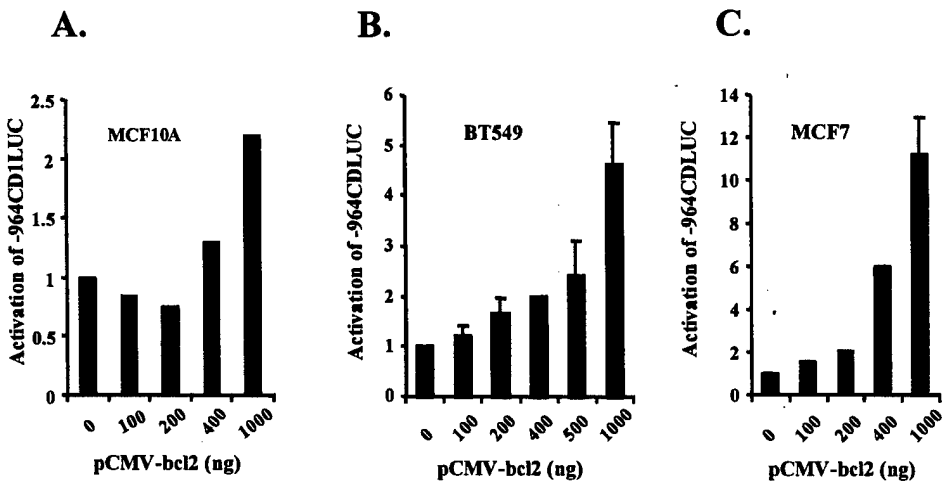


Fig. 2. Bcl-2 induces cyclin D_1 promoter activity in MCF10A, BT549 and MCF-7 cells. Three μg of –964CDLUC plasmid, 0.15 μg of pMDV-lacZ and increasing amounts of bcl-2 expression vector were co-transfected into MCF10A (A), BT549 (B) or MCF7 (C) cells. The plasimd pCRII (purchased from Stratagene) was supplemented to use the same amount of DNA in each transfection. Cell lysate was harvested 30 hours after transfection and the luciferase activity in 1μ g of cell lysate was normalized to β-galactosidase activity to control the transfection efficiencies. The luciferase activity in cells tranfected without bcl-2 expression plasmid was arbitrarily given as 1.

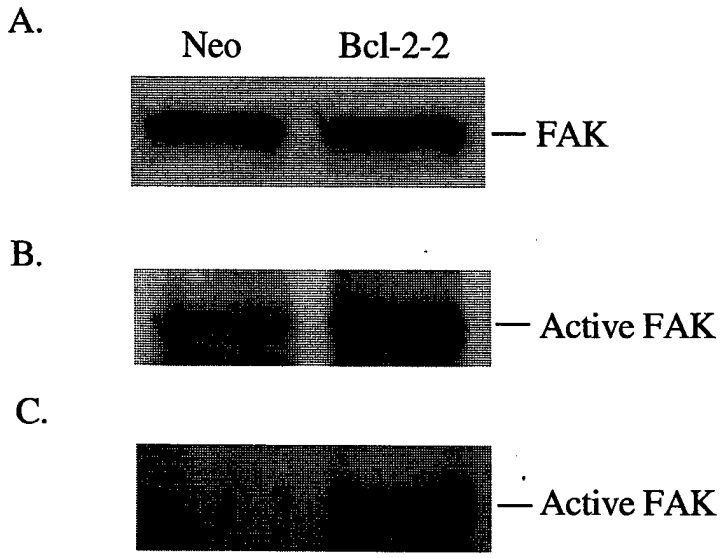


Fig.3 Bcl-2 constitutively activates focal adhesion kinase regardless of cell adhesion. (**A**) Lysates (50 μg/lane) of control (Neo) or bcl-2 overexpressing MCF10A clone 2 (Bcl-2-2) were subjected to immunoblot analysis using an anti-FAK mAb and detection by ECL. (**B**, **C**). were cultured (12 h) 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.

Table 1. Bcl-2 activation of cyclin D_1 promoter activity independent of cell anchorage

	Monolayer		Suspension	
	Control	pCMV-bcl-2	Control	pCMV-bcl-2
BT549	1	1.89±0.12	0.88±0.08	2.63±1.63
MCF-7	1	5.62±2.33	1.16±0.10	6.36±1.76
MCF10A	1	1.20±0.02	0.23±0.01	0.89±0.01

Table 1. Bcl-2 activation of cyclin D_1 promoter activity independent of cell anchorage. MCF10A, BT549, and MCF7 cells were transiently transfected for 24 hours with 3 μg of–964CDLUC, 0.15 μg of pMDV-lacZ and 0.5 μg of bcl-2 expression vector (pCMV-bcl-2) or control vector (control). Cells were trypsinized and cultured in suspension or monolayer for additional 24 hours before harvesting for luciferase activity assay. Data represent the average ± S.D. of 8 independent transfection experiments.

(9) Conclusion

Increasing evidence suggests that overexpression of cyclin D1 contributes to the oncogenic transformation of cells in vitro and in vivo (9-11). Involvement of bcl-2 in cancer development was believed to result from its ability to prevent cell death (thereby increasing cell number). However, our previous and present studies suggest that bcl-2 may serve as an oncogene in the development of human breast cancer, which involves induction of cyclin D1 expression.

We originally proposed to determine the in vivo oncogenic activity of bcl-2 using bcl-2 overexpressing MCF10ATG3B (we previously showed that bcl-2 overexpression in MCF10ATG3B induces a transformed phenotype as determined by a soft agar assay). Since the Career Development Award does not support any research expense, it was not feasible to perform the animal experiments. Instead, we have been investigating the roles of bcl-2 in the regulation of gene expression that are critical for apoptosis regulation and oncogenic activity.

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