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13. ABSTRACT ***(Maximum 200 Words)***

Galectin-3 is a multifunctional oncogenic protein found in the nucleus, cytoplasm and also extracellular milieu. Although recent studies demonstrated an anti-apoptotic activity of galectin-3, neither the functional site nor the mechanism of how galectin-3 regulates apoptosis is known. During the funding period, we examined the subcellular localization of galectin-3 during apoptosis and investigated its anti-apoptotic actions. Confocal microscopy and biochemical analysis revealed that galectin-3 is enriched in the mitochondria and prevents mitochondrial damage and cytochrome c release. Using a yeast two-hybrid system we screened for galectin-3 interacting proteins that regulate galectin-3 localization and anti-apoptotic activity. Synexin, a Ca^{2+}- and phospholipid-binding protein, was one of the proteins identified. We confirmed direct interaction between galectin-3 and synexin by GST-pulldown assay in vitro. We showed that galectin-3 fails to translocate to the perinuclear membranes when the expression of synexin is downregulated using an oligodeoxyribonucleotide complementary to the synexin mRNA, suggesting a role for synexin in galectin-3 trafficking. Furthermore, synexin downregulation abolished anti-apoptotic activity of galectin-3. Taken together, our study suggests that synexin mediates galectin-3 translocation to the perinuclear mitochondrial membranes, where it regulates mitochondrial integrity critical for apoptosis regulation.

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(4) Introduction

Galectin-3 is a 31 kDa member of the β-galactoside-binding family of proteins found widely in epithelial and immune cells. The expression of galectin-3 is associated with neoplastic progression and metastatic potential in human cancer cells including breast cancer (1-5). Galectin-3 modulates a variety of cellular processes. Extracellular galectin-3 mediates cell migration, cell adhesion and cell-cell interactions, while nuclear galectin-3 is involved in pre-mRNA splicing (6-8). Interestingly, recent studies showed that cytoplasmic galectin-3 is associated with tumor progression (9). Yet, the role of cytoplasmic galectin-3 is unknown.

We and others have previously shown that galectin-3 inhibits T-cell apoptosis induced by anti-Fas antibody and epithelial cell apoptosis induced by staurosporine, cisplatin, genistein, and anoikis (10-12). The anti-apoptotic activity of galectin-3 was also demonstrated in galectin-3 deficient mice. Peritoneal macrophages from galectin-3 deficient mice were more sensitive to apoptotic stimuli than those from control mice (13). The ability of galectin-3 to protect cells against apoptosis induced by agents working through different mechanisms suggests that galectin-3 regulates the common apoptosis commitment step.

The goals of the proposed study were (1) to determine the functional site of galection-3 for its anti-apoptotic action in human breast epithelial cells, and (2) to investigate the role of galectin-3 in the regulation of the signal transduction pathways during anoikis (apoptosis induced by loss of cell adhesion).

(4) Body of Report

4-1: Specific Aim 1 : To determine the functional site of galection-3 for its anti-apoptotic action in human breast epithelial cells

MATERIALS AND METHODS

Cell culture and reagents.

The human breast cancer cell line BT549 was obtained from Dr. E. W. Thompson, Vincent T. Lombardi Cancer Research Center, Georgetown University Medical Center (Washington, D. C.). Galectin-3 transfected BT549 cells (BT549Gal-3) were previously established by introducing an expression vector containing human galectin-3 cDNA into BT549 parental cells (11,12). The neo-resistant control vector transfected BT549 cells are referred to as BT549neo. Cells were cultured using DMEM/F12 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 0.5 μg/ml fungizone in a 95% air and 5% CO_2 incubator at 37^{0}C. All cell culture reagents were purchased from Invitrogen inc. (CA, USA)

DEVDase activity assay

Cells were lysed in CEB (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT) containing 0.03% Nonidet P-40. Lysates were centrifuged at 15,000 g for 10 min, and 50 μl cytosol fraction was incubated for 60 min at 37 ^{0}C in a total volume of 200 μl caspase buffer [10 mM HEPES (pH 7.5), 50 mM NaCl, and 2.5 mM DTT] containing 25 μM acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (Ac-DEVD-AMC, Bachem, PA). Using Spectra Maxi Germini fluorescence plate reader (Molecular Devices, CA), AMC fluorescence, released by caspase activity, was measured at 460 nm using 360 nm excitation wavelength. Caspase activity was normalized per microgram of protein determined by BCA protein assay kit (Pierce, IL).

Mitochondria staining

Cells were plated on a coverslip in a 12-well plate. After 24 hr of apoptosis induction, the cells were incubated with media containing 250 nM MitoTracker Red (Molecular Probes, Eugene, OR) for 30 minutes at 37^{0}C. Cells were washed with PBS, fixed with 3.7% paraformaldehyde in PBS for 15 min at 37^{0}C. The coverslips were mounted onto glass slides with anti-fade solution (Molecular Probes, OR). Fluorescent staining of the mitochondrial membrane was examined with a Nikon Labophot microscope fitted with a digital video

camera (Photometrics, Tucson, AZ) or a Zeiss LSM 310 microscope (Carl Zeiss, Germany) in the confocal mode.

Cytochrome c release

Cells were harvested at 0, 24, and 48 hr following treatment with 25 μM cisplatin, resuspended in ice-cold CEB (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 4 mM dithiothreitol) containing 250 mM sucrose and Protease Inhibitor cocktail (Roche, Mannheim, Germany), and incubated for 1 hr at 4^0C. The lysates were then passed through a 26 1/2 gauge syringe 15 times and then centrifuged at 15,000 g for 20 min at 4^0C. The resulting supernatant was analyzed by immunoblot analysis using anti-cytochrome c antibody (ZYMED Laboratories Inc, CA). The intensity of the bands was quantified using Biorad Quantity One program.

Confocal immunofluorescence microscopic analysis

Cells were cultured on coverslips to 75% confluency. Apoptosis was induced by treatment with 25 μM cisplatin for 24 hr or 0.5 μM staurosporine for 150 minutes, or by growth factor withdrawal for 48 hrs. Cells were washed with PBS three times, fixed with 3.7% formaldehyde in PBS for 15 minutes, washed with PBS-S (PBS-0.1% saponin), and then incubated with 1% BSA in PBS-S for 1 hr. After 6-washes with PBS-S, cells were incubated with rat anti-galectin-3 antibody (ATCC, VA) or anti-cytochrome c antibody (clone 6H2.B4, BD PharMingen, CA) for 2 hours at room temperature. After 6-washes with PBS-S, the cover slip was incubated with FITC conjugated secondary antibodies (Sigma, MO) for 1 hour. After 6-washes with PBS-S, the cover slip was mounted up-side-down with anti-fade solution (Molecular Probes, OR), sealed, and examined under a Zeiss LSM 310 microscope in the confocal mode.

Immunoblot analysis

Cell lysates were prepared using 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 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 minutes and subjected to reducing SDS-PAGE. After electrophoresis, the proteins were transferred to a nitrocellulo membrane. The blot was blocked with 5% nonfat dry milk in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.02% N and 0.2% Tween-20 (T-TBS) for 1 hour at room temperature. The membranes were incubated with the appropriate primary antibody in 5% milk in T-TBS. After three washes with T-TBS, the blot was incubated with the appropria HRP-conjugated secondary antibody. The antigen was detected using the ECL detection system (Pierce, IL) accor to the manufacturer's instruction.

Isolation of Mitochondria

Mitochondria were isolated as previously described (14). Briefly, BT549Gal-3 cells were homogenized in CEB with 250 mM sucrose to protect mitochondria by 20 strokes using a type B dauncer (Knotes Glass Company, NJ). Homogenates were centrifuged at 750 g for 3 X 10 min at 4 ^{0}C to remove debris and nuclei. Then the supernatant was centrifuged at 15,000g for 20 min, the pellet, which contains mitochondria, was lysed in SDS lysis buffer and 20 μg mitochondrial proteins were subjected to immunoblot analysis.

A cell-free caspase activation system

Cells were lysed in CEB using a Douncer as previously described (15). The protein concentration of the lysate was measured using BCA protein assay kit and adjusted to 4 μg/μl. To activate caspase, cytochrome c (purified from bovine heart, Sigma, MO) was added into cell-free extracts to a final concentration of 50 μg/ml and incubated at 37^0C. DEVDase activity was measured as described above.

Construction of the bait plasmid for yeast two-hybrid screening.

The yeast expression vector pEG202 (16), which contains the coding sequences for the LexA DNA-binding domain (amino acids 1-202) and yeast *HIS3* gene, was used to express the bait fusion proteins. The galectin-3 cDNA insert containing the full-length coding sequences was excised from previously constructed plasmid pcDNA10-Galectin-3 (11) and fused in-frame to the LexA DNA binding domain. The correct orientation and in-frame fusion were confirmed by DNA sequencing. Expression of the fusion proteins was confirmed by immunoblot analysis. The bait plasmid containing LexA and galectin-3 fusion protein was designated as pLG52.

Yeast media and strains

All yeast media were prepared as described (17). Minimal dropout media contains either 2% glucose (Glu) or 2% galactose (Gal) plus 2% raffinose (Raf). The dropout media lacks uracil, histidine, tryptophan, or leucine and are designated as -ura, -his, -urp, or -leu respectively. Minimal media containing 0.16 mg/mL (X-Gal) was used to test the *lacZ* reporter gene expression. YPD media contains yeast extract, peptone, and 2% glucose. The bait plasmid pLG52 was introduced into *Saccharomyces cerevisiae* yeast strains RFY206 (MATa *his*3Δ200 *leu*2-3*lys*2Δ201 *ura*3-52 *trp*1Δ::*his*G) (18) containing a *lacZ* reporter plasmid pSH18-34 (17) with yeast URA3 gene by LiOAc-mediated transformation (17). Transformants were selected by growth on Glu -ura -his dropout medium. The expression of the fusion protein was confirmed by immunoblot analysis using both anti-LexA and anti-galectin-3 antibodies. To test whether the bait alone would activate the reporter gene *LEU2*, the bait strain was mated to RFY231 (MATα *trp1*Δ::*hisG his3 ura3-1 leu2*::3Lexop-*LEU2*) (19), containing the *TRP1* vector pJG4-5 (16) which is also used to express the cDNA library. The number of diploids were grown on Gal/Raf -ura -his -trp -leu (Leu^+ colony) and Gal/Raf -ura -his –trp (colony-forming units, CFU) plates were counted. The ratio of Leu^+ colonies vs. total CFU was 7.5×10^{-7}, indicating that the background was low and the bait plasmid was suitable for yeast two-hybrid screening.

Yeast two-hybrid screening.

The human prostate tumor cDNA library cloned into the pJG4-5 plasmid was obtained from OriGene Technology Inc (MD,USA) and maintained in yeast RFY231. The bait strain was mated to the RFY231/pJG4-5 cDNA library as previously described (19,20). Out of 2×10^7 diploid colony-forming units, 244 Leu^+ were selected. Leu^+ colonies were printed onto four indicator plates: Glu -ura-his-trp-leu; Gal/Raf –ura-his-trp-leu; Glu X-Gal -ura-his-trp; and Gal/Raf X-Gal –ura-his-trp-. The colonies that showed galactose-dependent Leu^+ and $lacZ^+$ phenotypes were further analyzed. Plasmids were rescued from the galactose-dependent Leu^+ and $lacZ^+$ colonies by a yeast mini-prep method as previously described (17). PCR amplification was performed using primers BCO1 (5'CCA GCC TCT TGC TGA GTG GAG ATG 3') and BCO2 (5' GAC AAG CCG ACA ACC TTG ATT GGA G 3') to amplify the insert. The PCR products were digested with restriction enzyme *Alu*I and *Hae*III to avoid sequencing identical clones. The mini-prep DNA was then transformed into *E.coli* KC8 and purified.

Specificity test

The purified prey plasmid DNA from the galactose-dependent Leu^+ and $lacZ^+$ colonies were introduced back into yeast strain RFY231, and transformants were mated to RFY206/pSH18-34 containing the bait plasmid pLG52 or other randomly chosen baits [LexA fusion bait plasmids pRFHM1 (21), plex202-hairy (22), p202-DmCdk4 (Finley, unpublished), pKL1 (Lavine, Finley, unpublished), pJG21-1 (16)]. Diploids were printed onto four indicator plates as above. The clones that specifically interact with pLG52 but not with other baits were sequenced. The sequences were analyzed using NCBI BLAST Search program

Preparation of recombinant galectin-3 protein.

LB medium containing 10 mM $MgCl_2$ and 100 μg/mL ampicillin was inoculated with an overnight culture of HMS-174 transformed with the plasmid containing the human galectin-3 cDNA insert. When the

bacterial cells were grown to an optical density of 0.5, IPTG (1 mM) was added. Cells were then incubated for an additional 4h and harvested by centrifugation at 1250g at 4°C. The pellet was washed with PBS and suspended in 100 mM of ice-cold lysis buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris, pH 8, 0.241 unit/mL aprotinin, 1μg/mL leupeptin, 1 μg/ml pepstatin, and 0.2 mM PMSF). The bacterial cells were disrupted by sonication and the lysate was centrifuged at 40,000g for 20 min and the supernatants were passed through an asialofetuin affinity column (100 x 22 mm inner diameter). The column was made by linking the asialofetuin to Affigel-15 (Bio-Rad, CA) according to the manufacturer's protocol, and was well equilibrated in phosphate buffer (10 mM phosphate, 1 mM MgSO4, 0.2 mM PMSF, and 0.2% NaN_3, pH 7.2). The column was washed with 3-5 column volumes of phosphate buffer, and the bound protein was eluted with 0.2M lactose. Eluted fractions were quantitated using BCA protein assay reagent (Pierce, IL), analyzed by SDS-PAGE and immunoblot analysis using anti-galectin-3 antibody.

Preparation of GST-synexin fusion protein and in vitro binding assay

The expression plasmid pGEX-KG-synexin for GST-synexin fusion protein was obtained from Dr. Creutz (University of Virginia). The GST expression plasmid pGST-4T3 was obtained from Dr. Brooks (Wayne State University). The GST and GST-synexin fusion proteins were prepared as previously described (23). Briefly, expression of GST and GST-synexin fusion proteins were induced in E. coli XL-1 Blue cells by 100 μM isopropyl-1-thio--D-galactopyranoside for 5 hrs. The cells were collected by centrifugation (5000 × g, 10 min), resuspended in 50 ml PBST1 (1xPBS with 1% Triton X-100) and lysed by sonication. The lysate was centrifuged at 10000 × g for 10 min. The supernatant was incubate with 1 ml (50% slurry) reduced Glutathione (GSH)-Sepharose (Amarsham Pharmacia, NJ). After washing the beads with PBS for 6 times, the binding proteins were eluted with 1 ml 10 mM reduced glutathione for 3 times. The eluted proteins were dialyzed against PBS overnight at 4 °C using Slide-A-Lyzer 10K dialysis cassette (Pierce, IL). In vitro binding of galectin-3 to synexin was examined by GST pulldown assay as previously described (24). Briefly, GSH–Sepharose beads were pretreated with bacterial lysates. 1 μg of GST or GST–synexin proteins in 500 μl PBST1 were absorbed to 50 μl of the pretreated beads (50% slurry) for 2 hrs, then the beads were washed with PBST1 3 times. Then, 1 μg of recombinant human galectin-3 proteins in 500 μl PBST05 (1 XPBS with 0.05% Triton X-100) were incubated with the GST or GST–synexin beads in the absence or presence of 20 μg of BSA at room temperature for 2 hr. In a control experiment, 1 μg of galectin-3 protein in 500 μl PBST05 was heat-denatured by boiling for 5 min followed by quenching on ice. After washing the beads with PBST05 for 6 times, the binding proteins were eluted with 25 μl SDS sample buffer and subjected to SDS-PAGE and immunoblot analysis.

Preparation of Antisense and Scrambled Oligonucleotides

The phosphorothioate oligonucleotide (5'-GGA TAG CCT GGG TAT GAC ATT C-3'), complementary to the human synexin mRNA sequences surrounding the ATG translation start-site, and the control oligonucleotides containing scrambled nucleotide sequences (5'-CTT ACA GTA TGG GTC CGA TAG G-3') were synthesized and purified by HPLC at Integrated DNA Technologies, Inc (IA, USA). To detect cells into which the oligomers are introduced, the antisense oligonucleotides were labeled with tetramethylrhodamine (TAMRA) at the 3'-end (Integrated DNA Technologies, Inc).

Transient transfection of oligonucleotides into BT549Gal-3 cells.

Cells were transfected with oligonucleotides using effectene as instructed by the manufacturer (Qiagen, CA). Briefly, 2 μg antisense or scrambled oligonucleotides were mixed with 120 μl EC buffer and 16 μl enhancer by vortexing for 1 second followed by incubation for 4 min at room temperature. The DNA was vortexed with 20 μl effectene for 1 minute, followed by incubation for 10 minutes. The DNA-effectene solution was mixed with 800 μl growth medium and overlaid onto cells grown in 6-well plates to 70% confluency. In control experiments, cells were treated with effectene mixture without oligonucleotides, or with antisense or scrambled oligonucleotides without effectene. After 24 hrs, the cells were harvested and lysed in

SDS lysis buffer for immunoblot analysis or in caspase lysis buffer for caspase activity assay. For transfection with TARMA-labeled antisense oligonucleotides, cells grown on a cover slip in a 12-well plate were treated with the DNA-effectene mixture (1 μg oligonucleotide, 60 μl EC buffer, 8 μl enhancer, 10 μl effectene and 400 μl media). After 6 hrs transfection, the media was replaced with fresh growth media and 24 hrs later the cells were treated with 0 and 0.5 μM staurosporine for 150 minutes and then stained with anti-galectin-3 antibody/FITC-conjugated 2nd antibody.

RESULTS

Galectin-3 protects mitochondrial integrity.

Mitochondrial events critical for apoptosis include the disruption of electron transport, loss of mitochondrial transmembrane potential (Ψ_m), and the release of cytochrome c (25,26), resulting in caspase activation. To examine whether galectin-3 protects mitochondrial integrity, we stained BT549neo and BT549Gal-3 cells with MitoTracker Red which selectively stains mitochondria and serves as a marker for the mitochondrial membrane potential (27). Overexpression of glaectin-3 in BT549Gal-3 cells was confirmed by immunoblot analysis (Fig. 1A, Appendix 1). Thirty-six hour treatment with 25 μM cisplatin resulted in the loss of mitochondrial structure in BT549neo cells (Fig. 1B and C, Appendix 1). In contrast, the mitochondria in cisplatin-treated BT549Gal-3 cells retained the fibrillar fluorescence pattern as observed in the untreated cells (Fig. 1D and E, Appendix 1), suggesting that galectin-3 overexpression protects cells against the loss of $\Delta\Psi_m$. As predicted from the loss of mitochondrial integrity, the immunoblot analysis of cytosolic cytochrome c showed that the level of cytochrome c released from the mitochondria was elevated in BT549neo cells as compared with BT549Gal-3 cells following cisplatin treatment (Fig. 1F, Appendix 1). After 48 hr treatment, more than 10 fold increase in cytosolic cytochrome c was detected in BT549neo cells, whereas only ~1.6 fold increase was observed in BT549Gal-3 cells. To exclude the possibility that cytochrome c was released to the cytosolic fractions during the preparation of cellular homogenates, we performed immunostaining of cytochrome c in control and apoptotic cells. The majority of BT549neo cells exhibited diffuse cytochrome c staining following treatment with cisplatin (Fig. 2B, Appendix 1) or staurosporine (Fig. 2D, Appendix 1), or after growth factor withdrawal (Fig. 2C, Appendix 1). In contrast, cytochrome c staining in BT549Gal-3 cells remained punctuate following the same treatment (Fig. 2F,G,H, Appendix 1). Confocal immunofluorescence microscopic analysis of cells co-stained with anti-cytochrome c Ab/ FITC conjugated secondary Ab and with MitoTracker Red confirmed that cytochrome c remained in the mitochondria in BT549Gal-3 cells following apoptotic stimuli, as shown by yellow staining (Fig. 2N,O,P, Appendix 1). These results showed that galectin-3 overexpression protects cells from losing their mitochondrial membrane potential and prevents cytochrome c release to the cytosol, following a variety of apoptotic stimuli.

Galectin-3 inhibition of cytochrome c release is critical for its inhibition of caspase activation.

We previously showed that galectin-3 overexpression results in inhibition of poly(ADP-ribose) polymerase (PARP) cleavage following apoptotic stimuli (11,12), suggesting that galectin-3 downregulates caspase activation. Since effector caspases (such as caspase-3, and -7) cleave PARP at the $DEVD^{216}$-G site, we measured cisplatin- and staurosporine-induced DEVDase activity in BT549neo and BT549Gal-3 cells using the fluorogenic substrate Ac-DEVD-AMC. DEVDase (caspase-3 like) activity increased ~5 fold at 48 hr following 25 μM cisplatin treatment in BT549neo cells, whereas it increased only ~ 2 fold following the same treatment in BT549Gal-3 (Fig. 3A, Appendix 1). Similarly, DEVDase activity increased ~ 5 fold at 2.5 hr following 0.5 μM staurosporine treatment in BT549neo cells, whereas no significant increase was detected in BT549Gal-3 cells (Fig. 3B, Appendix 1). These results showed that the caspase-3 (effector caspase)-like activity necessary for apoptosis execution is significantly inhibited by galectin-3 overexpression. We then tested whether galectin-3 inhibition of caspase activity results from changes in the apoptotic machinery necessary for caspase activation, or from inhibition of cytochrome c release. To this end, we established a cell free-caspase activation system as previously described (15). Caspases in extracts from human breast epithelial cells were effectively activated by adding

cytochrome c at 50 μg/ml, and the activation kinetics were comparable between BT549neo and BT549-Gal-3 cell-free extracts (Fig. 3C, Appendix 1). Surprisingly, cell-free extracts from BT549Gal-3 contained caspases that can be activated at even higher levels in the presence of cytochrome c compared to the BT549neo cell extracts. These results indicate that BT549Gal-3 cells contain all the necessary components to activate caspases, and suggest that galectin-3 downregulation of caspases largely results from its ability to inhibit cytochrome c release following apoptotic stimuli.

Galectin-3 is redistributed onto the intracellular membranes following apoptotic stimuli.

To determine the subcellular location where galectin-3 exerts its anti-apoptotic effect, galectin-3 protein in the control and apoptotic cells was stained with anti-galectin-3 mAb and FITC conjugated secondary Ab. Galectin-3 staining was evenly detected in the nucleus and the cytoplasm in the control BT549Gal-3 cells (Fig. 4A, Appendix 1). In contrast, galectin-3 staining displayed a compact and punctuated extranuclear membrane staining in cells treated with cisplatin (Fig. 4B, Appendix 1), cultured in serum-free medium (Fig. 4C, Appendix 1) or treated with staurosporine (Fig. 4D, Appendix 1). This suggests that following apoptotic stimuli, galectin-3 translocates into the intracellular membrane, possibly mitochondria where it prevents mitochondrial dysfunction and inhibits caspase activation. To examine whether galectin-3 translocates to the mitochondria, cells were co-stained with anti-galectin-3 mAb and with MitoTracker Red. The galectin-3 staining patterns (Fig. 4B, C, D, Appendix 1) strikingly resembled the mitochondrial staining patterns (Fig. 4F, G, H, Appendix 1) following apoptotic stimuli, but not in the control cells (Fig. 4A and E, Appendix 1). Confocal microscopic analysis from the same plane of focus revealed that galectin-3 and mitochondria indeed co-localize following apoptotic stimuli as shown by yellow staining (Fig. 4J, K, L, Appendix 1). To further confirm galectin-3 translocation to the mitochondria, mitochondria were isolated in the presence of 250 mM sucrose as previously described (14). Immunoblot analysis confirmed a significant increase in the level of galectin-3 protein in the mitochondrial fraction following apoptotic stimuli (Fig. 4M, Appendix 1). To ensure the

cytochrome c oxidase

Galectin-3 interacts with synexin.

The galectin-3 protein lacks signal sequences for its subcellular localization, suggesting that galectin-3 translocation may occur through its interaction with other proteins that direct protein trafficking. To search for proteins that interact with full-length galectin-3, we screened a human prostate tumor cDNA library using the LexA yeast two-hybrid screening methods as described (16,17,19). Out of 2 X 10^7 diploid colony-forming units screened, 17 positive colonies were detected. cDNA plasmids were isolated from the positive colonies and amplified in *E. coli* for further analyses. The purified plasmids were introduced back into yeast and tested for specific interactions by performing two-hybrid interaction mating assays with the LexA-galectin-3 bait or other 5 randomly chosen baits. Eleven of the 17 candidates interacted with galectin-3 but not with 5 randomly chosen baits. DNA sequencing analysis revealed that 2 of the 11 prey plasmids encoded for the full-length synexin protein. Yeast two-hybrid interaction mating assays showed specific interactions between galectin-3 and synexin (Fig. 5A, Appendix 1). The remaining 9 represented 2 novel proteins that will be described elsewhere.

To confirm the direct interaction between galectin-3 and synexin, we performed an *in vitro* binding assay. GST and GST-synexin fusion proteins were purified from the bacterial expression system, and direct interactions between synexin and recombinant galectin-3 proteins were tested by GST pull-down assays as described in Materials and Methods. As shown in Fig. 5B in Appendix 1, Galectin-3 bound directly to GST-synexin but not to GST. Synexin effectively interacted with galectin-3 in the presence of large excess of bovine serum albumin, whereas it failed to bind to heat-denatured galectin-3 proteins, indicating that galectin-3 binding to synexin is specific.

Synexin is critical for galectin-3 translocation to the perinuclear membrane and apoptosis regulation.

Synexin (Annexin VII) is a member of the Annexin, Ca^{2+}- and phospholipid-binding family of the proteins (28). Synexin is thought to act as a Ca^{2+} channel and as a Ca^{2+}-activated GTPase, thus regulating

Ca^{2+}/GTPase-dependent secretory events (29,30). To examine the significance of synexin for galectin-3 trafficking and apoptosis regulation, we downregulated synexin expression in BT549Gal-3 cells using antisense oligonucleotides. Transfection of the oligonucleotides complementary to the synexin mRNA significantly downregulated synexin expression, whereas control oligonucleotides (scrambled sequences) had no effect (Fig. 6, Appendix 1). When synexin expression is downregulated, intracellular galectin-3 levels also decreased while extracellular galectin-3 levels increased (~ 5 fold), suggesting that synexin is involved in galectin-3 trafficking.

We then examined whether synexin expression is critical for subcellular redistribution of galectin-3 following apoptotic stimuli. To detect cells into which oligonucleotides are introduced, cells were transfected with the antisense oligonucleotides labeled with tetramethylrhodamine (TAMRA) at the 3'-end. Immunostaining with anti-galectin-3 antibody/FITC conjugated 2^{nd} antibody showed that galectin-3 staining was evenly detected in the nucleus and the cytoplasm in antisense oligonucleotides-transfected BT549Gal-3 cells (Fig. 7A, C, Appendix 1) as in control BT549Gal-3 cells (Fig. 4A, Appendix 1). Cells with no or small amounts of antisense oligonucleotide contained high levels of intracellular galectin-3 proteins (indicated by long arrows in Fig. 7A, C, Appendix 1), whereas cells with high levels of the antisense oligomers retained lower levels of intracellular galectin-3 (indicated by short double arrows in Fig. 7A, C, Appendix 1). These are consistent with the results obtained by immunoblot analysis showing that synexin downregulation results in downregulation of intracellular galectin-3 levels (Fig. 6, Appendix 1). Following apoptotic stimuli, galectin-3 is localized to the perinuclear membranes in BT549Gal-3 cells (Fig. 4D, Appendix 1), whereas it remained evenly distributed in cells transfected with antisense oligonucleotides (Fig. 7B, Appendix 1). Consistently, redistribution of galectin-3 proteins was shown in BT549 cells into which antisense oligonucletides were introduced at low efficiency (inserts in Fig. 7B,D, Appendix 1). These results indicate that synexin expression is critical for intracellular galectin-3 expression and redistribution during apoptosis. We then tested whether disruption of galectin-3 translocation by downregulation of synexin has effects on the anti-apoptotic activity of galectin-3. We measured staurosporine-induced DEVDase activity in BT549Gal-3 cells with or without synexin downregulation (Fig. 7E, Appendix 1). The basal levels of DEVDase activity in BT549Gal-3 cells were not significantly altered by synexin downregulation using anti-sense oligonucleotides. However, DEVDase activity increased more than 3-fold following staurosporine when synexin expression is downregulated. This increase was similar to the level of DEVDase activation in BT549neo cells following the same treatment (Fig. 3B, Appendix 1), showing that synexin downregulation abolished galectin-3's ability to downregulate caspase activity.

4-2: Specific Aim 2 : To investigate the role of galectin-3 in the regulation of the signal transduction pathways during anoikis.

While BT549 cells (human breast epithelial cells) undergo anoikis, galectin-3 overexpressing BT549 cells respond to the loss of cell adhesion by inducing G_1 arrest without detectable cell death (12). Galectin-3 mediated G_1 arrest involves downregulation of G_1-S cyclin levels (cyclin E and cyclin A) and upregulation of their inhibitory protein levels ($p21^{WAF1/CIP1}$ and $p27^{KIP1}$). Following the loss of cell anchorage, Rb protein becomes hypophosphorylated in galectin-3 overexpressing cells, as predicted from the flow cytometric analysis and immunoblot analysis of cyclins and their inhibitors. Interestingly, galectin-3 induces cyclin D_1 expression (an early G_1 cyclin) and its associated kinase activity in the absence of cell anchorage. Based on these results, we proposed that galectin-3 inhibition of anoikis involves cell cycle arrest at an anoikis-insensitive point (late G_1) through modulation of gene expression and activities of cell cycle regulators. Since cyclin D_1 a critical regulator of the cell cycle during anoikis and a potential oncogene in breast cancer, we investigated the role of galectin-3 on regulation of cyclin D_1 gene expression. We found that galectin-3 induces cyclin D_1 promoter activity in human breast epithelial cells independent of cell adhesion through multiple cis-elements, including the SP1 and CRE sites. We presented evidence that galectin-3 induction of the cyclin D_1 promoter may result from enhancement/stabilization of nuclear protein-DNA complex formation at the CRE site of the cyclin D_1

promoter. We also showed that galectin-3 co-operates with, but does not depend on, pRb for cyclin D_1 promoter activation. The results of these studies are presented in **Appendix 2.**

(6) Key Research Accomplishments

During the funding period of 1999-2002, we investigated the functional site of galectin-3 for its inhibition of apoptosis, and the role of galectin-3 in the regulation of cyclin D_1 gene expression during anoikis. We also expanded our study on galectin-3 regulation of apoptosis induced by genistein and nitric-oxide.

We found that following a variety of apoptotic stimuli, galectin-3 translocates to the perinuclear membrane and prevents mitochondrial damage and cytochrome c release, thereby effectively protecting cells against apoptosis induced by agents working through different apoptosis initiation pathways (**Appendix 1**).

We previously showed that galectin-3 inhibition of anoikis involves cell cycle arrest at an anoikis-insensitive point (late G_1) through modulation of gene expression and activities of cell cycle regulators including cyclin D_1. We found that galectin-3 induces cyclin D_1 promoter activity in human breast epithelial cells independent of cell adhesion through multiple cis-elements, including the SP1 and CRE sites. Although galectin-3 regulation of cyclin D_1 expression is unlikely to be a common mechanism for galectin-3 inhibition of apoptosis, it may be critical for anoikis in which cell cycle regulation is critical for apoptosis regulation (**Appendix 2**).

We also found that galectin-3 is a potent inhibitor of apoptosis induced a variety of stimuli including genistein and nitric-oxide (**Appendix 3 and 4**).

(7) Reportable Outcomes

Publications:

Lin, H-M., Moon, B-K., Yu, F. and **Kim, H.-R. C.** Galectin-3 mediates genistein-induced G_2/M arrest and inhibits apoptosis. Carcinogenesis 21: 1941-1945, 2000

Moon, B.-K., Lee, Y. J., Battle, P., Jessup, J. M., Raz, A. and **Kim, H.-R. C**. Galectin-3 protects human breast carcinoma cells against nitric oxide-induced apoptosis: Implication of galectin-3 function during metastasis. Am. J. Pathology 159: 1055-1060, 2001

Yoshii, T., Honjo, Y., Inohara, H., **Kim, H.-R. C.**, and Raz, A. Galectin-3 phosphorylation modulates its biological functions: Anti-apoptosis and cell cycle arrest. J. Biol. Chem. 277: 6852-6857, 2002

Yu, F., Finley, R. L. Jr., Raz, A. and **Kim, H.-R. C**. Galectin-3 translocates to the perinuclear membranes and inhibits cytochrome c release from the mitochondria: A role for synexin in galectin-3 translocation. J. Biol. Chem. 277: 15819-15827, 2002

Lin, H-M., Pestell, R. G., Raz, A. and **Kim, H.-R. C.** Galectin-3 enhances cyclin D_1 promoter activity through SP1 and a cAMP-responsive element in human breast epithelial cells. Oncogene, In press

(8) Conclusion

Galectin-3 is a critical regulator of the apoptosis commitment step involving mitochondria and caspase cascade

in human breast epithelial cells.

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Galectin-3 Translocates to the Perinuclear Membranes and Inhibits Cytochrome *c* Release from the Mitochondria

A ROLE FOR SYNEXIN IN GALECTIN-3 TRANSLOCATION*

Appendix 1

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Galectin-3 is a multifunctional oncogenic protein found in the nucleus and cytoplasm and also the extracellular milieu. Although recent studies demonstrated an anti-apoptotic activity of galectin-3, neither the functional site nor the mechanism of how galectin-3 regulates apoptosis is known. In this study, we examined the subcellular localization of galectin-3 during apoptosis and investigated its anti-apoptotic actions. We report that galectin-3 translocates to the perinuclear membrane following a variety of apoptotic stimuli. Confocal microscopy and biochemical analysis revealed that galectin-3 is enriched in the mitochondria and prevents mitochondrial damage and cytochrome *c* release. Using a yeast two-hybrid system, we screened for galectin-3-interacting proteins that regulate galectin-3 localization and anti-apoptotic activity. Synexin, a Ca^{2+}- and phospholipid-binding protein, was one of the proteins identified. We confirmed direct interaction between galectin-3 and synexin by glutathione *S*-transferase pull-down assay *in vitro*. We showed that galectin-3 failed to translocate to the perinuclear membranes when expression of synexin was down-regulated using an oligodeoxyribonucleotide complementary to the synexin mRNA, suggesting a role for synexin in galectin-3 trafficking. Furthermore, synexin down-regulation abolished anti-apoptotic activity of galectin-3. Taken together, these results suggest that synexin mediates galectin-3 translocation to the perinuclear mitochondrial membranes, where it regulates mitochondrial integrity critical for apoptosis regulation.

Galectin-3 is a 31-kDa member of the β-galactoside-binding family of proteins found widely in epithelial and immune cells. Expression of galectin-3 is associated with neoplastic progression and metastatic potential (1–5) in head and neck (6), thyroid (7), gastric (3), and colon (8) cancers, suggesting a role in oncogenesis. Galectin-3 modulates a variety of cellular processes. Extracellular galectin-3 mediates cell migration, cell adhesion, and cell/cell interactions, whereas nuclear galectin-3 is involved in pre-mRNA splicing (9–11). Interestingly, recent studies showed that cytoplasmic, but not nuclear, galectin-3 is associated with tumor progression (12, 13). Yet, the role of cytoplasmic galectin-3 is unknown.

We (15–17) and others (14, 18, 19) have previously shown that galectin-3 inhibits T-cell apoptosis induced by anti-Fas antibody and epithelial cell apoptosis induced by staurosporine, cisplatin, genistein, and anoikis. The anti-apoptotic activity of galectin-3 was also demonstrated in galectin-3-deficient mice. Peritoneal macrophages from galectin-3-deficient mice were more sensitive to apoptotic stimuli than those from control mice (20). The ability of galectin-3 to protect cells against apoptosis induced by agents working through different mechanisms suggests that galectin-3 regulates the common apoptosis commitment step.

During the past decade, explosive progress has been made toward understanding the molecular basis for the regulation of the apoptosis commitment step. Two major apoptotic pathways (intrinsic and extrinsic pathways) have been defined. Intrinsic apoptotic signaling induces cytochrome *c* release from the mitochondria. Cytosolic cytochrome *c* initiates the formation of an ~700-kDa complex called the "apoptosome," which consists of cytochrome *c*, caspase adaptor proteins such as Apaf-1, and caspases (21–23). Apoptosome formation results in caspase activation, a commitment step for apoptosis induction. Extrinsic apoptotic signals are mediated by cell-surface death receptors, including Fas, tumor necrosis factor, and TRAIL receptor families. The death domain of the death receptor initiates the formation of the "death-inducing signaling complex," where caspases are activated (reviewed in Ref. 24). Although a critical role for galectin-3 in apoptosis inhibition is now well documented, neither the functional site nor the molecular mechanism of how galectin-3 regulates apoptosis is understood.

In this study, we investigate the subcellular localization of galectin-3 and its anti-apoptotic actions during intrinsic apoptosis in human breast epithelial cells (BT549). Here, we report that galectin-3 translocates to the perinuclear mitochondrial membranes and inhibits cytochrome *c* release following a variety of apoptotic stimuli. Whereas caspase activation is drastically down-regulated by galectin-3 overexpression in BT549 cells, exogenous cytochrome *c* effectively activates caspases in a cell-free system established from galectin-3-overexpressing cells. This suggests that galectin-3 protection of mitochondrial integrity is critical for its ability to down-regulate caspases. We identified synexin as a galectin-3-interacting protein using the yeast two-hybrid system and provide evidence that synexin is involved in galectin-3 translocation to the functional site for its anti-apoptotic actions.

MATERIALS AND METHODS

Cell Culture and Reagents—The human breast cancer cell line BT549 was obtained from Dr. E. W. Thompson (Vincent T. Lombardi Cancer

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Research Center, Georgetown University Medical Center, Washington, D. C.). Galectin-3-transfected BT549 cells (BT549Gal-3) were previously established by introducing an expression vector containing human galectin-3 cDNA into parental BT549 cells (15, 16). The neomycin-resistant control vector-transfected BT549 cells are referred to as BT549neo. Cells were cultured using Dulbecco's modified Eagle's medium/nutrient mixture F-12 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 0.5 μg/ml Fungizone in a 95% air and 5% CO_2 incubator at 37 °C. All cell culture reagents were purchased from Invitrogen.

DEVDase[1] Activity Assay—Cells were lysed in cell extract buffer (CEB) (20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol) containing 0.03% Nonidet P-40. Lysates were centrifuged at 15,000 × *g* for 10 min, and 50 μl of the cytosolic fraction was incubated for 60 min at 37 °C in a total volume of 200 μl of caspase buffer (10 mM HEPES (pH 7.5), 50 mM NaCl, and 2.5 mM dithiothreitol) containing 25 μM acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Bachem, King of Prussia, PA). Using a Spectra Maxi Germini fluorescence plate reader (Molecular Devices, Menlo Park, CA), 7-amino-4-methylcoumarin fluorescence, released by caspase activity, was measured at 460 nm using 360-nm excitation wavelength. Caspase activity was normalized per microgram of protein determined by the BCA protein assay kit (Pierce).

Mitochondrial Staining—Cells were plated on coverslips in 12-well plates. After 24 h of apoptosis induction, the cells were incubated with medium containing 250 nM MitoTracker Red (Molecular Probes, Inc., Eugene, OR) for 30 min at 37 °C. Cells were washed with PBS and fixed with 3.7% paraformaldehyde in PBS for 15 min at 37 °C. The coverslips were mounted onto glass slides with anti-fade solution (Molecular Probes, Inc.). Fluorescent staining of the mitochondrial membrane was examined with a Nikon Labophot microscope fitted with a digital video camera (Photometrics Ltd., Tucson, AZ) or a Zeiss LSM 310 microscope in the confocal mode.

Cytochrome c Release—Cells were harvested at 0, 24, and 48 h following treatment with 25 μM cisplatin; resuspended in ice-cold CEB containing 250 mM sucrose and protease inhibitor mixture (Roche Molecular Biochemicals, Mannheim, Germany); and incubated for 1 h at 4 °C. The lysates were then passed through a 26½-gauge syringe 15 times and then centrifuged at 15,000 × *g* for 20 min at 4 °C. The resulting supernatant was analyzed by immunoblot analysis using anti-cytochrome *c* antibody (Zymed Laboratories Inc., South San Francisco, CA). The intensity of the bands was quantified using the Bio-Rad Quantity One program.

Confocal Immunofluorescence Microscopic Analysis—Cells were cultured on coverslips to 75% confluency. Apoptosis was induced by treatment with 25 μM cisplatin for 24 h or with 0.5 μM staurosporine for 150 min or by growth factor withdrawal for 48 h. Cells were washed with PBS three times, fixed with 3.7% formaldehyde in PBS for 15 min, washed with PBS-S (PBS containing 0.1% saponin), and then incubated with 1% bovine serum albumin in PBS-S for 1 h. After six washes with PBS-S, cells were incubated with rat anti-galectin-3 antibody (American Type Culture Collection, Manassas, VA) or anti-cytochrome *c* antibody (clone 6H2.B4, BD PharMingen) for 2 h at room temperature. After six washes with PBS-S, the coverslips were incubated with FITC-conjugated secondary antibodies (Sigma) for 1 h. After six washes with PBS-S, the coverslips were mounted upside down with anti-fade solution, sealed, and examined under a Zeiss LSM 310 microscope in the confocal mode.

Immunoblot Analysis—Cell lysates were prepared using SDS lysis buffer (2% SDS, 125 mM Tris-HCl (pH 6.8), and 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 the BCA protein assay reagent (Pierce). Equal amounts of protein samples in SDS sample buffer (1% SDS, 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, and 0.05% bromphenol 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 blocked with 5% nonfat dry milk in Tris-buffered saline/Tween (100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.02% NaN_3, and 0.2% Tween 20) for 1 h at room temperature. The membranes were incubated with the appropriate primary antibody in 5% milk in Tris-buffered saline/Tween. After three washes with Tris-buffered saline/Tween, the blot was incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. The antigen was detected using the ECL detection system (Pierce) according to the manufacturer's instruction.

Isolation of Mitochondria—Mitochondria were isolated as previously described (25). Briefly, BT549Gal-3 and BT549neo cells were homogenized in CEB containing 250 mM sucrose to protect mitochondria by 20 strokes using a type B Dounce homogenizer (Kontes Glass Co., Vineland, NJ). Homogenates were centrifuged at 750 × *g* for 3 × 10 min at 4 °C to remove debris and nuclei. The supernatant was then centrifuged at 15,000 × *g* for 20 min; the pellet, which contained mitochondria, was lysed in SDS lysis buffer; and 20 μg of mitochondrial proteins was subjected to immunoblot analysis.

A Cell-free Caspase Activation System—Cells were lysed in CEB using a Dounce homogenizer as previously described (26). The protein concentration of the lysate was measured using the BCA protein assay kit and adjusted to 4 μg/μl. To activate caspase, cytochrome *c* (purified from bovine heart; Sigma) was added to cell-free extracts to a final concentration of 50 μg/ml and incubated at 37 °C. DEVDase activity was measured as described above.

Construction of the Bait Plasmid for Yeast Two-hybrid Screening—The yeast expression vector pEG202 (27), which contains the coding sequences for the LexA DNA-binding domain (amino acids 1–202) and the yeast *HIS3* gene, was used to express the bait fusion proteins. The galectin-3 cDNA insert containing the full-length coding sequences was excised from the previously constructed plasmid pcDNA10-galectin-3 (15) and fused in-frame with the LexA DNA-binding domain. The correct orientation and in-frame fusion were confirmed by DNA sequencing. Expression of the fusion proteins was confirmed by immunoblot analysis. The bait plasmid containing the LexA-galectin-3 fusion protein was designated as pLG52.

Yeast Media and Strains—All yeast media were prepared as described (28). Minimal dropout media contained either 2% glucose (Glc) or 2% galactose (Gal) plus 2% raffinose (Raf). The dropout media lacked uracil, histidine, tryptophan, or leucine and are designated as −Ura, −His, −Trp, or −Leu, respectively. Minimal medium containing 0.16 mg/ml X-gal was used to test *lacZ* reporter gene expression. YPD medium contained yeast extract, peptone, and 2% dextrose. The bait plasmid pLG52 was introduced into *Saccharomyces cerevisiae* yeast strain RFY206 (*MATa his3Δ200 leu2-3lys2Δ201 ura3-52 trp1Δ::hisG*) (29) containing the *lacZ* reporter plasmid pSH18-34 (28) with the yeast *URA3* gene by LiOAc-mediated transformation (28). Transformants were selected by growth on Glc−Ura−His dropout medium. Expression of the fusion protein was confirmed by immunoblot analysis using both anti-LexA and anti-galectin-3 antibodies. To test whether the bait alone would activate the reporter gene *LEU2*, the bait strain was mated with RFY231 (*MATα trp1Δ::hisG his3 ura3-1 leu2*::3Lexop-*LEU2*) (30) containing the *TRP1* vector pJG4-5 (27), which was also used to express the cDNA library. The number of diploids grown on Gal/Raf−Ura−His−Trp−Leu (Leu$^+$ colony) and Gal/Raf−Ura−His−Trp (colony-forming units) plates was counted. The ratio of Leu$^+$ colonies *versus* total colony-forming units was 7.5×10^{-7}, indicating that the background was low and that the bait plasmid was suitable for yeast two-hybrid screening.

Yeast Two-hybrid Screening—The human prostate tumor cDNA library cloned into the pJG4-5 plasmid was obtained from OriGene Technology Inc. (Rockville, MD) and maintained in yeast strain RFY231. The bait strain was mated with the RFY231/pJG4-5 cDNA library as previously described (30, 31). Out of 2×10^7 diploid colony-forming units, 244 Leu$^+$ colonies were selected. Leu$^+$ colonies were printed onto four indicator plates: Glc−Ura−His−Trp−Leu, Gal/Raf−Ura−His−Trp−Leu, Glc/X-gal−Ura−His−Trp, and Gal/Raf/X-gal−Ura−His−Trp. The colonies that showed galactose-dependent Leu$^+$ and LacZ$^+$ phenotypes were further analyzed. Plasmids were rescued from the galactose-dependent Leu$^+$ and LacZ$^+$ colonies by a yeast mini-prep method as previously described (28). PCR amplification was performed using primers BCO1 (5′-CCA GCC TCT TGC TGA GTG GAG ATG-3′) and BCO2 (5′-GAC AAG CCG ACA ACC TTG ATT GGA G-3′) to amplify the insert. The PCR products were digested with restriction enzymes *Alu*I and *Hae*III to avoid sequencing identical clones. The mini-prep DNA was then transformed into *Escherichia coli* KC8 and purified.

Specificity Test—The purified prey plasmid DNAs from the galactose-dependent Leu$^+$ and LacZ$^+$ colonies were introduced back into yeast strain RFY231, and transformants were mated with RFY206/pSH18-34 containing the bait plasmid pLG52 or other randomly chosen baits (LexA fusion bait plasmids pRFHM1 (32), pLex202-hairy (33), p202-

[1] The abbreviations used are: DEVDase, (Asp-Glu-Val-Asp)ase; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; Raf, raffinose; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; GST, glutathione *S*-transferase.

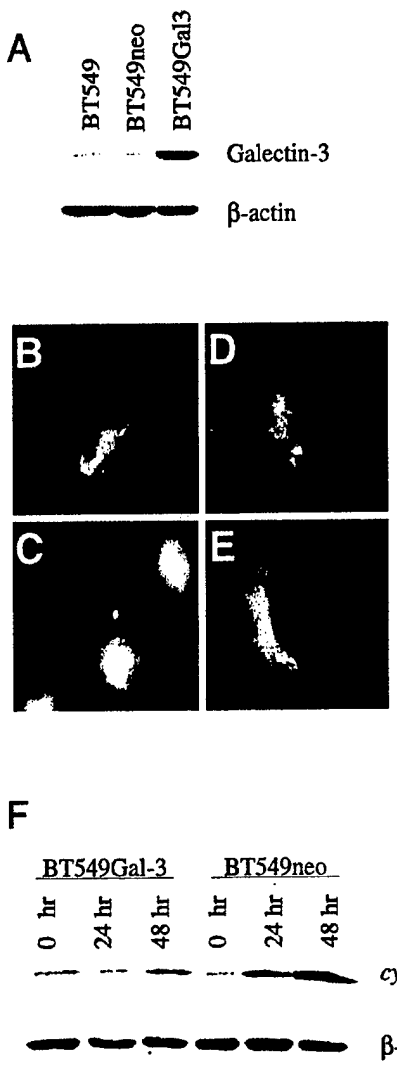


FIG. 1. **Galectin-3 expression in human breast epithelial cells prevents mitochondrial damage and cytochrome *c* release.** *A*, immunoblot analysis of galectin-3 was performed using 20 μg of cell lysates from parental BT549, BT549neo, and BT549Gal-3 cells. The same blot was reprobed with anti-β-actin antibody to confirm the equal loading of proteins in each lane. *B–E*, BT549neo (*B* and *C*) and BT549Gal-3 (*D* and *E*) cells were cultured on coverslips and treated with 0 (*B* and *D*) or 25 (*C* and *E*) μM cisplatin. After 36 h, the cells were stained with a fluorescent probe for the mitochondrial membrane potential (MitoTracker Red). The fluorescence study was carried out with a Nikon Labophot microscope fitted with a digital video camera. *F*, immunoblot analysis of cytosolic cytochrome *c* (*cyt c*) was performed using cytosolic proteins prepared from BT549Gal-3 and BT549neo cells treated with or without 25 μM cisplatin for 24 or 48 h. To confirm the equal loading of proteins in each lane, the same blot was reprobed with anti-β-actin antibody.

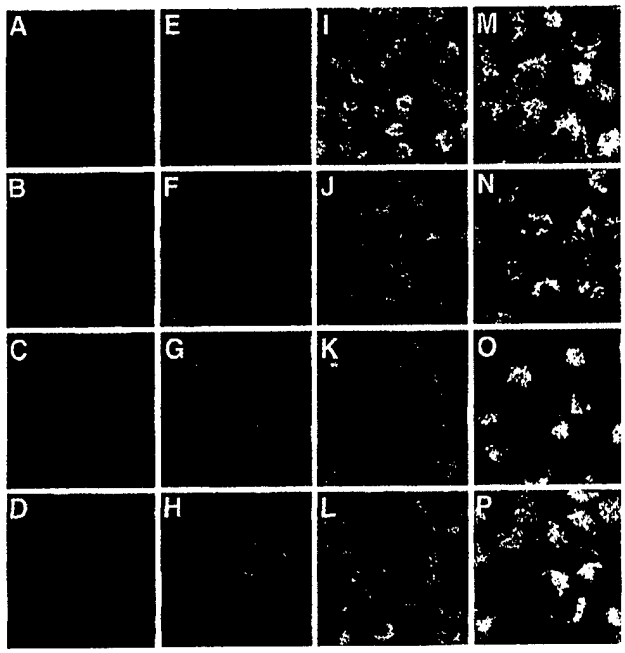


FIG. 2. **Galectin-3 expression in human breast epithelial cells inhibits cytochrome *c* release from mitochondria in response to apoptotic stimuli.** BT549neo (*A–D* and *I–L*) and BT549Gal-3 (*E–H* and *M–P*) cells were cultured on coverslips with no treatment (*A*, *E*, *I*, and *M*), with 25 μM cisplatin for 24 h (*B*, *F*, *J*, and *N*), with serum-free medium for 48 h (*C*, *G*, *K*, and *O*), or with 0.5 μM staurosporine for 150 min (*D*, *H*, *L*, and *P*). The mitochondria and cytochrome *c* were stained as described under "Materials and Methods." *A–H* show cytochrome *c* staining only; *I–P* are composite images of cytochrome *c* (*green*) and mitochondria (*red*), with the *yellow* color indicating co-localization.

DmCdk4,[2] pKL1,[3] and pJG21-1 (27)). Diploids were printed onto four indicator plates as described above. The clones that specifically interacted with pLG52 but not with other baits were sequenced. The sequences were analyzed using the NCBI BLAST search program.

Preparation of Recombinant Galectin-3 Protein—LB medium containing 10 mM $MgCl_2$ and 100 μg/ml ampicillin was inoculated with an overnight culture of HMS-174 cells transformed with the plasmid containing the human galectin-3 cDNA insert. When the bacterial cells were grown to an absorbance of 0.5, isopropyl-1-thio-β-D-galactopyranoside (1 mM) was added. Cells were then incubated for an additional 4 h and harvested by centrifugation at 1250 × *g* at 4 °C. The pellet was washed with PBS and suspended in 100 mM ice-cold lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8), 0.241 units/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.2 mM phenylmethylsulfonyl fluoride). The bacterial cells were disrupted by sonication; the lysate was centrifuged at 40,000 × *g* for 20 min; and the supernatants were passed through an asialofetuin affinity column (22, inner diameter, × 100 mm). The column was made by linking the asialofetuin to Affi-Gel 15 (Bio-Rad) according to the manufacturer's protocol and was well equilibrated in phosphate buffer (10 mM phosphate, 1 mM $MgSO_4$, 0.2 mM phenylmethylsulfonyl fluoride, and 0.2% NaN_3 (pH 7.2)). The column was washed with 3–5 column volumes of phosphate buffer, and the bound protein was eluted with 0.2 M lactose. Eluted fractions were quantitated using the BCA protein assay reagent and analyzed by SDS-PAGE and immunoblot analysis using anti-galectin-3 antibody.

Preparation of the GST-Synexin Fusion Protein and in Vitro Binding Assay—The expression plasmid pGEX-KG-synexin for the GST-synexin fusion protein was obtained from Dr. Carl E. Creutz (University of Virginia). The GST expression plasmid pGST-4T3 was obtained from Dr. Brooks (Wayne State University). The GST and GST-synexin fusion proteins were prepared as previously described (34). Briefly, expression of the GST and GST-synexin fusion proteins was induced in *E. coli* XL-1Blue cells by 100 μM isopropyl-1-thio-β-D-galactopyranoside for 5 h. The cells were collected by centrifugation (5000 × *g*, 10 min), resuspended in 50 ml of PBST1 (1× PBS with 1% Triton X-100), and lysed by sonication. The lysate was centrifuged at 10000 × *g* for 10 min. The supernatant was incubated with 1 ml (50% slurry) of reduced GSH-Sepharose (Amersham Biosciences). After washing the beads six times with PBS, the binding proteins were eluted three times with 1 ml of 10 mM reduced glutathione. The eluted proteins were dialyzed against PBS overnight at 4 °C using Slide-A-Lyzer 10K dialysis cassette (Pierce). *In vitro* binding of galectin-3 to synexin was examined by GST pull-down assay as previously described (35). Briefly, GSH-Sepharose beads were pretreated with bacterial lysates. 1 μg of GST or GST-synexin proteins in 500 μl of PBST1 was absorbed to 50 μl of the pretreated beads (50% slurry) for 2 h, and then the beads were washed three times with PBST1. Then, 1 μg of recombinant human galectin-3 proteins in 500 μl of PBST05 (1× PBS with 0.05% Triton X-100) was incubated with the GST or GST-synexin beads in the absence or presence of 20 μg of bovine serum albumin at room temperature for 2 h. In a control experiment, 1 μg of galectin-3 protein in 500 μl of PBST05 was heat-denatured by boiling for 5 min, followed by quenching on ice. After

[2] R. L. Finley, Jr., unpublished data.
[3] K. L. Lavine and R. L. Finley, Jr., unpublished data.

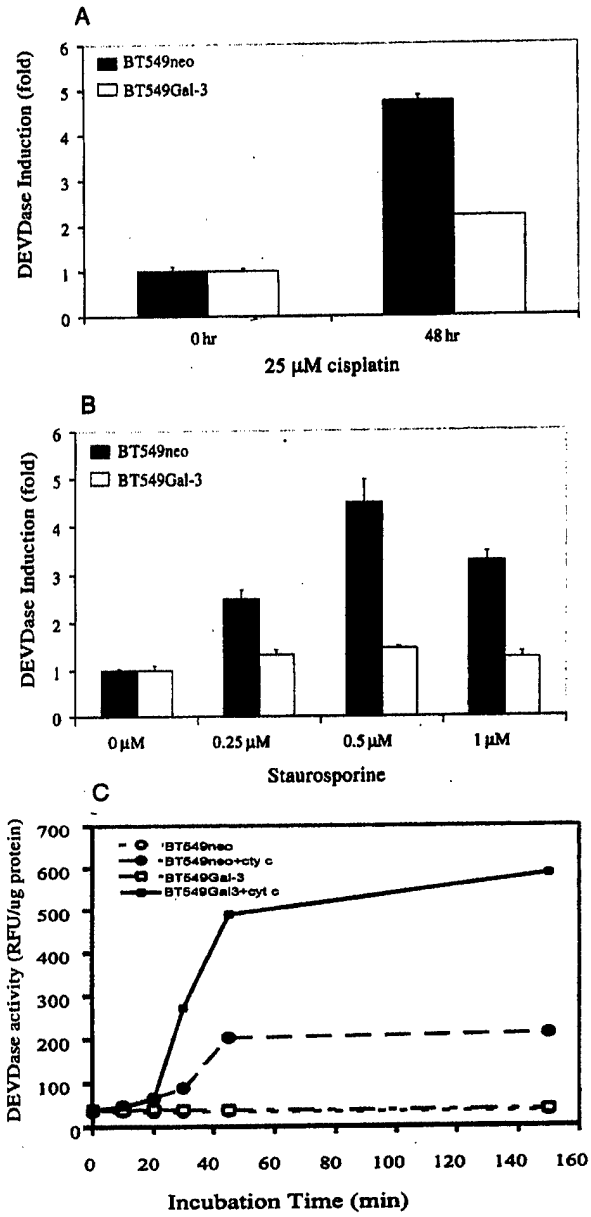


FIG. 3. **Galectin-3 expression in human breast epithelial cells down-regulates DEVDase activity.** *A* and *B*, BT549neo and BT549Gal-3 cells were treated with 25 μM cisplatin for 48 h (*A*) or with staurosporine for 150 min (*B*). DEVDase activities were measured and normalized per microgram of protein. Three independent experiments were performed, and the *error bars* represent the mean ± S.D. of triplicates. The level of DEVDase activity in untreated cells was arbitrarily given as 1. *C*, cell-free caspase activation systems were established from BT549neo and BT549Gal-3 cells as described under "Materials and Methods." Cell-free extracts of BT549neo and BT549Gal-3 cells were incubated with or without exogenous cytochrome *c* (*cyt c*; 50 μg/ml) for the indicated time periods, and DEVDase activities were measured. *RFU*, relative fluorescence units.

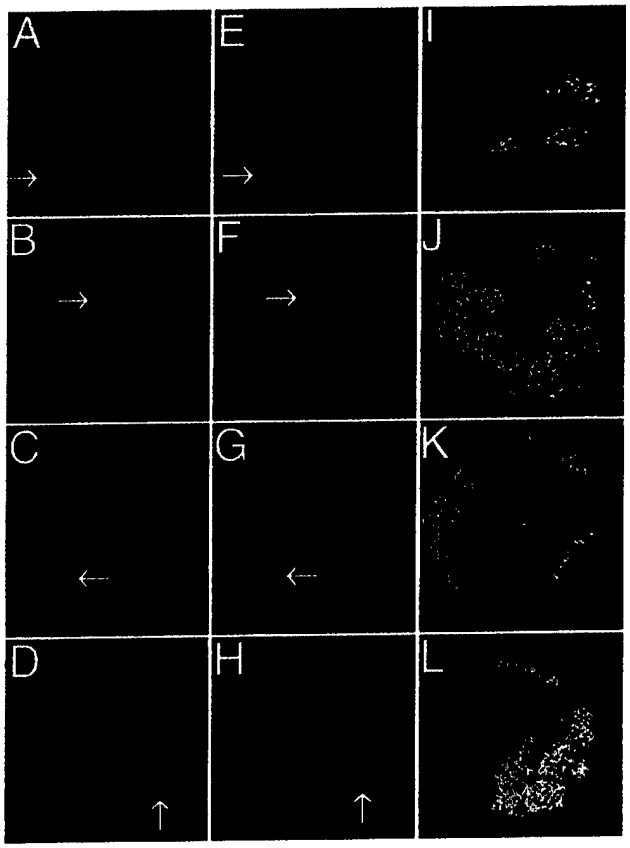
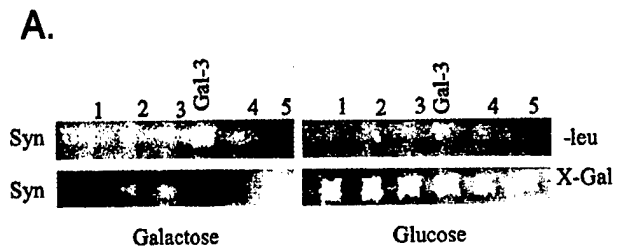


FIG. 4. **Galectin-3 is redistributed onto the intracellular membranes following apoptotic stimuli.** *A–L*, BT549Gal-3 cells treated with no apoptotic stimulus (*A*, *E*, and *I*), with 25 μM cisplatin for 24 h (*B*, *F*, and *J*), with serum-free medium for 48 h (*C*, *G*, and *K*), or with 0.5 μM staurosporine for 150 min (*D*, *H*, and *L*) were co-stained with Mito-Tracker Red and anti-galectin-3 antibody/FITC-conjugated secondary antibody. Galectin-3 (*green* staining; *A–D*) and mitochondria (*red* staining; *E–H*) are shown. Co-localization of galectin-3 and mitochondria (*yellow* staining; *I–L*) is shown by composite images of cells (indicated by *arrows*) at a higher magnification. *M*, mitochondria were isolated from BT549Gal-3 cells treated with no apoptotic stimulus (control (*Ctr*)), with serum-free medium for 48 h (*SF*), with 25 μM cisplatin for 24 h (*Cisp*), or with 0.5 μM staurosporine for 150 min (*STS*). The levels of galectin-3 proteins in the mitochondria were detected by immunoblot analysis (*upper panel*). To confirm the equal loading of the mitochondrial proteins in each lane, the same blot was probed with anti-cytochrome *c* (*cyt c*) oxidase antibody (Molecular Probes, Inc.) (*lower panel*).

washing the beads six times with PBST05, the binding proteins were eluted with 25 μl of SDS sample buffer and subjected to SDS-PAGE and immunoblot analysis.

Preparation of Antisense and Scrambled Oligonucleotides—The phosphorothioate oligonucleotide (5′-GGA TAG CCT GGG TAT GAC ATT C-3′), complementary to the human synexin mRNA sequences surrounding the ATG translation start site, and the control oligonucleotides containing scrambled nucleotide sequences (5′-CTT ACA GTA TGG GTC CGA TAG G-3′) were synthesized and purified by high performance liquid chromatography at Integrated DNA Technologies, Inc. (Coralville, IA). To detect cells into which the oligomers were introduced, the antisense oligonucleotides were labeled with tetramethylrhodamine at the 3′-end (Integrated DNA Technologies, Inc.).

Transient Transfection of Oligonucleotides into BT549Gal-3 Cells—Cells were transfected with oligonucleotides using Effectene (QIAGEN Inc., Chatsworth, CA) as instructed by the manufacturer. Briefly, 2 μg of antisense or scrambled oligonucleotides was mixed with 120 μl of EC buffer and 16 μl of enhancer by vortexing for 1 s, followed by incubation for 4 min at room temperature. The DNA was vortexed with 20 μl of Effectene for 1 min, followed by incubation for 10 min. The DNA/Effectene solution was mixed with 800 μl of growth medium and overlaid onto cells grown in six-well plates to 70% confluency. In control experiments, cells were treated with the Effectene mixture without oligonucleotides or with antisense or scrambled oligonucleotides without Effectene. After 24 h, the cells were harvested and lysed in SDS lysis buffer for immunoblot analysis or in caspase lysis buffer for caspase activity assay. For transfection with tetramethylrhodamine-labeled antisense oligonucleotides, cells grown on a coverslip in a 12-



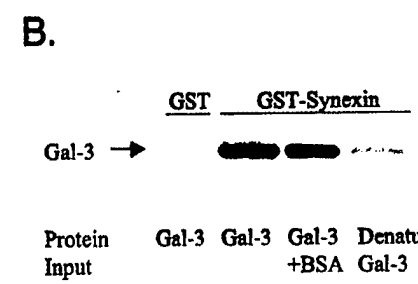


FIG. 5. **Galectin-3 interacts with synexin.** *A*, yeast two-hybrid interaction mating assay. Yeast RFY206/pSH18-34 containing the LexA-galectin-3 bait (*Gal-3*) or five randomly chosen baits (pRFHM1 (32) (*lane 1*), plex202-hairy (*lane 2*), p202-DmCdk4 (*lane 3*), pKL1 (*lane 4*), or pJG21-1 (*lane 5*)) was mated with RFY231 containing the synexin prey. Diploids were replicated onto −Ura−His−Trp−Leu medium (*−leu*; *upper panels*) or −Ura−His−Trp medium containing X-gal (*X-Gal*; *lower panels*). The *left panels* contained galactose, and the *right panels* contained glucose. The specific interaction between galectin-3 and synexin (*Syn*) was detected by galactose-dependent growth on the −Leu plate and by galactose-dependent β-galactosidase expression as indicated by the *blue* colony on the X-gal plate. *B*, GST pull-down assay. 1 μg of GST (*first lane*) or GST-synexin fusion proteins (*second* through *fourth lanes*) bound to GSH-Sepharose beads were incubated with 1 μg of recombinant human galectin-3 proteins (*Gal-3*) in the absence (*first* and *second lanes*) or presence (*third lane*) of 20 μg of bovine serum albumin (*BSA*) or with 1 μg of heat-denatured galectin-3 proteins (*fourth lane*). Galectin-3 proteins eluted from the beads were detected by immunoblot analysis using anti-galectin-3 antibody.

well plate were treated with the DNA/Effectene mixture (1 μg of oligonucleotide, 60 μl of EC buffer, 8 μl of enhancer, 10 μl of Effectene, and 400 μl of medium). After 6 h of transfection, the medium was replaced with fresh growth medium; and 24 h later, the cells were treated with 0 and 0.5 μM staurosporine for 150 min and then stained with anti-galectin-3 antibody/FITC-conjugated secondary antibody.

RESULTS

Galectin-3 Protects Mitochondrial Integrity—Mitochondrial events critical for apoptosis include the disruption of electron transport, loss of mitochondrial transmembrane potential, and release of cytochrome *c* (23, 36), resulting in caspase activation. To examine whether galectin-3 protects mitochondrial integrity, we stained BT549neo and BT549Gal-3 cells with MitoTracker Red, which selectively stains mitochondria and serves as a marker for the mitochondrial membrane potential (37). Overexpression of galectin-3 in BT549Gal-3 cells was confirmed by immunoblot analysis (Fig. 1*A*). Thirty-six hours of treatment with 25 μM cisplatin resulted in the loss of mitochondrial structure in BT549neo cells (Fig. 1, *B* and *C*). In contrast, the mitochondria in cisplatin-treated BT549Gal-3 cells retained the fibrillar fluorescence pattern, as observed in the untreated cells (Fig. 1, *D* and *E*), suggesting that galectin-3 overexpression protects cells against the loss of mitochondrial transmembrane potential. As predicted from the loss of mitochondrial integrity, the immunoblot analysis of cytosolic cytochrome *c* showed that the level of cytochrome *c* released from the mitochondria was elevated in BT549neo cells compared with BT549Gal-3 cells following cisplatin treatment (Fig. 1*F*).

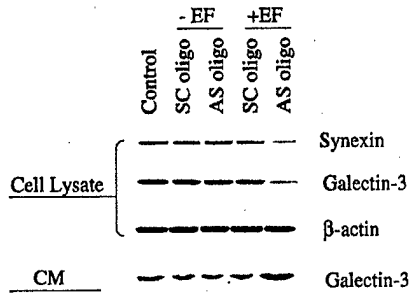
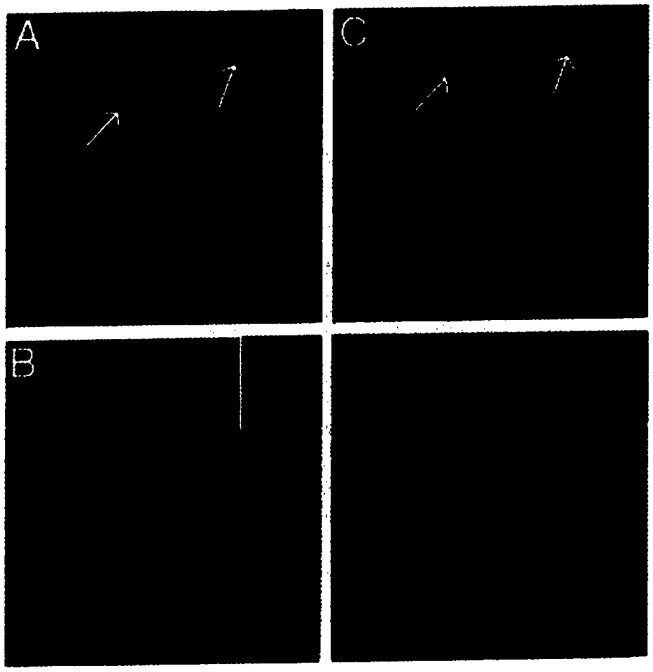


FIG. 6. **Synexin expression is critical for galectin-3 trafficking.** BT549Gal-3 cells were treated with Effectene without oligonucleotides (*Control*) or with scrambled oligonucleotides (*SC oligo*) or antisense oligonucleotides complementary to the synexin mRNA (*AS oligo*) in the absence (*−EF*) or presence of (*+EF*) Effectene for 24 h. The synexin and galectin-3 levels in total cell lysates were detected by immunoblot analysis using anti-synexin and anti-galectin-3 antibodies, respectively. To confirm the equal loading of proteins in each lane, the same blot was probed with anti-β-actin antibody. Extracellular galectin-3 levels were detected by immunoblot analysis using conditioned medium (*CM*).

After 48 h of treatment, a >10-fold increase in cytosolic cytochrome *c* was detected in BT549neo cells, whereas only an ~1.6-fold increase was observed in BT549Gal-3 cells. To exclude the possibility that cytochrome *c* was released to the cytosolic fractions during the preparation of cellular homogenates, we performed immunostaining of cytochrome *c* in control and apoptotic cells. The majority of BT549neo cells exhibited diffuse cytochrome *c* staining following treatment with cisplatin (Fig. 2*B*) or staurosporine (Fig. 2*D*) or after growth factor withdrawal (Fig. 2*C*). In contrast, cytochrome *c* staining in BT549Gal-3 cells remained punctate following the same treatment (Fig. 2, *F–H*). Confocal immunofluorescence microscopic analysis of cells co-stained with anti-cytochrome *c* antibody/FITC-conjugated secondary antibody and with MitoTracker Red confirmed that cytochrome *c* remained in the mitochondria in BT549Gal-3 cells following apoptotic stimuli, as shown by *yellow* staining (Fig. 2, *N–P*). These results show that galectin-3 overexpression protects cells from losing their mitochondrial membrane potential and prevents cytochrome *c* release to the cytosol following a variety of apoptotic stimuli.

Galectin-3 Inhibition of Cytochrome c Release Is Critical for Its Inhibition of Caspase Activation—We previously showed that galectin-3 overexpression results in inhibition of poly-(ADP-ribose) polymerase cleavage following apoptotic stimuli (15, 16), suggesting that galectin-3 down-regulates caspase activation. Because effector caspases (such as caspase-3 and -7) cleave poly(ADP-ribose) polymerase at the $DEVD^{216}G$ site, we measured cisplatin- and staurosporine-induced DEVDase activity in BT549neo and BT549Gal-3 cells using the fluorogenic substrate acetyl-DEVD-7-amino-4-methylcoumarin. DEVDase (caspase-3-like) activity increased ~5-fold at 48 h following 25 μM cisplatin treatment in BT549neo cells, whereas it increased only ~2-fold following the same treatment in BT549Gal-3 (Fig. 3*A*). Similarly, DEVDase activity increased ~5-fold at 2.5 h following 0.5 μM staurosporine treatment in BT549neo cells, whereas no significant increase was detected in BT549Gal-3 cells (Fig. 3*B*). These results show that the caspase-3 (effector caspase)-like activity necessary for apoptosis execution is significantly inhibited by galectin-3 overexpression. We then tested whether galectin-3 inhibition of caspase activity results from changes in the apoptotic machinery necessary for caspase activation or from inhibition of cytochrome *c* release. To this end, we established a cell-free caspase activation system as previously described (26). Caspases in extracts from human breast epithelial cells were effectively activated by adding cytochrome *c* at 50 μg/ml, and the activation kinetics were comparable between BT549neo and BT549-Gal-3 cell-free extracts



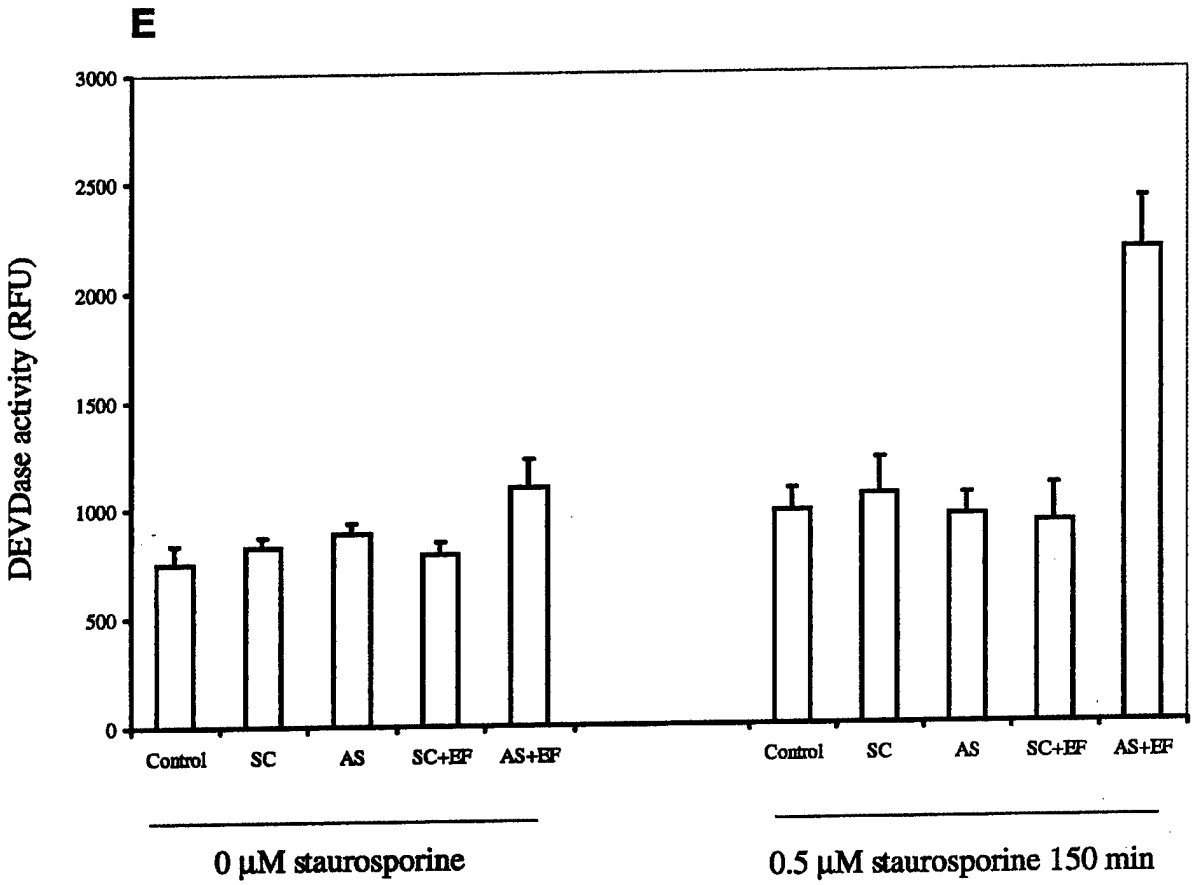


FIG. 7. **Synexin expression is critical for galectin-3 translocation and apoptosis regulation in human breast epithelial cells.** *A–D*, BT549Gal-3 cells transfected with tetramethylrhodamine-labeled antisense synexin oligonucleotides were treated with 0 (*A* and *C*) or 0.5 μM staurosporine for 150 min (*B* and *D*), stained with anti-galectin-3 antibody/FITC-conjugated secondary antibody, and examined under a confocal microscope. Galectin-3 (*green* staining) and transfected tetramethylrhodamine-labeled oligonucleotides (*red* staining) are shown. In *A* and *C*, *single-tailed arrows* indicate cells with low levels of tetramethylrhodamine-labeled oligonucleotides, and *double-tailed arrows* indicate cells with high levels of oligonucleotides. The *insets* in *B* and *D* represent a staurosporine-treated cell into which tetramethylrhodamine-labeled oligonucleotides were introduced at a low level. *E*, BT549Gal-3 cells were treated with Effectene without oligonucleotides (*Control*), with scrambled oligonucleotides without Effectene (*SC*), with antisense oligonucleotides without Effectene (*AS*), with scrambled oligonucleotides with Effectene

(Fig. 3*C*). Surprisingly, cell-free extracts from BT549Gal-3 cells contained caspases that could be activated at even higher levels in the presence of cytochrome *c* compared with the BT549neo cell extracts. These results indicate that BT549Gal-3 cells contain all the necessary components to activate caspases and suggest that galectin-3 down-regulation of caspases largely results from its ability to inhibit cytochrome *c* release following apoptotic stimuli.

Galectin-3 Is Redistributed onto the Intracellular Membranes following Apoptotic Stimuli—To determine the subcellular location where galectin-3 exerts its anti-apoptotic effect, the galectin-3 protein in the control and apoptotic cells was stained with anti-galectin-3 monoclonal antibody and FITC-conjugated secondary antibody. Galectin-3 staining was evenly detected in the nucleus and cytoplasm in the control BT549Gal-3 cells (Fig. 4*A*). In contrast, galectin-3 staining displayed a compact and punctate extranuclear membrane staining in cells treated with cisplatin (Fig. 4*B*), cultured in serum-free medium (Fig. 4*C*), or treated with staurosporine (Fig. 4*D*). This suggests that following apoptotic stimuli, galectin-3 translocates to the intracellular membrane, possibly to the mitochondria, where it prevents mitochondrial dysfunction and inhibits caspase activation. To examine whether galectin-3 translocates to the mitochondria, cells were co-stained with anti-galectin-3 monoclonal antibody and MitoTracker Red. The galectin-3 staining patterns (Fig. 4, *B–D*) strikingly resembled the mitochondrial staining patterns (Fig. 4, *F–H*) following apoptotic stimuli, but not those in the control cells (Fig. 4, *A* and *E*). Confocal microscopic analysis from the same plane of focus revealed that galectin-3 and mitochondria indeed co-localized following apoptotic stimuli, as shown by *yellow* staining (Fig. 4, *J–L*). To further confirm galectin-3 translocation to the mitochondria, mitochondria were isolated in the presence of 250 mM sucrose as previously described (25). Immunoblot analysis confirmed a significant increase in the level of galectin-3 protein in the mitochondrial fraction following apoptotic stimuli (Fig. 4*M*).

Galectin-3 Interacts with Synexin—The galectin-3 protein lacks signal sequences for its subcellular localization, suggesting that galectin-3 translocation may occur through its interaction with other proteins that direct protein trafficking. To search for proteins that interact with full-length galectin-3, we screened a human prostate tumor cDNA library using the LexA yeast two-hybrid screening methods as described (27, 28, 30). Out of 2×10^7 diploid colony-forming units screened, 17 positive colonies were detected. cDNA plasmids were isolated from the positive colonies and amplified in *E. coli* for further analyses. The purified plasmids were introduced back into yeast cells and tested for specific interactions by performing two-hybrid interaction mating assays with the LexA-galectin-3 bait or with five other randomly chosen baits. Eleven of the 17 candidates interacted with galectin-3, but not with the five randomly chosen baits. DNA sequencing analysis revealed that 2 of the 11 prey plasmids encoded for the full-length synexin protein. Yeast two-hybrid interaction mating assays showed specific interactions between galectin-3 and synexin (Fig. 5*A*). The remaining nine prey plasmids represented two novel proteins that will be described elsewhere.

To confirm the direct interaction between galectin-3 and synexin, we performed an *in vitro* binding assay. GST and GST-synexin fusion proteins were purified from the bacterial expression system, and direct interactions between synexin and recombinant galectin-3 proteins were tested by GST pull-down assays as described under "Materials and Methods." As shown in Fig. 5*B*, galectin-3 bound directly to GST-synexin, but not to GST. Synexin effectively interacted with galectin-3 in the presence of a large excess of bovine serum albumin, whereas it failed to bind to heat-denatured galectin-3 proteins, indicating that galectin-3 binding to synexin is specific.

Synexin Is Critical for Galectin-3 Translocation to the Perinuclear Membrane and Apoptosis Regulation—Synexin (annexin VII) is a member of the annexin Ca^{2+}- and phospholipid-binding family of proteins (38). Synexin is thought to act as a Ca^{2+} channel and as a Ca^{2+}-activated GTPase, thus regulating Ca^{2+}/GTPase-dependent secretory events (39, 40). To examine the significance of synexin for galectin-3 trafficking and apoptosis regulation, we down-regulated synexin expression in BT549Gal-3 cells using antisense oligonucleotides. Transfection of the oligonucleotides complementary to the synexin mRNA significantly down-regulated synexin expression, whereas control oligonucleotides (scrambled sequences) had no effect (Fig. 6). When synexin expression was down-regulated, intracellular galectin-3 levels also decreased, whereas extracellular galectin-3 levels increased (~5-fold), suggesting that synexin is involved in galectin-3 trafficking.

We then examined whether synexin expression is critical for subcellular redistribution of galectin-3 following apoptotic stimuli. To detect cells into which oligonucleotides were introduced, cells were transfected with the antisense oligonucleotides labeled with tetramethylrhodamine at the 3′-end. Immunostaining with anti-galectin-3 antibody/FITC-conjugated secondary antibody showed that galectin-3 staining was evenly detected in the nucleus and cytoplasm in antisense oligonucleotide-transfected BT549Gal-3 cells (Fig. 7, *A* and *C*) as in control BT549Gal-3 cells (Fig. 4*A*). Cells with no or small amounts of the antisense oligonucleotide contained high levels of intracellular galectin-3 proteins (Fig. 7, *A* and *C*, *single-tailed arrows*), whereas cells with high levels of the antisense oligomers retained lower levels of intracellular galectin-3 (*double-tailed arrows*). These results are consistent with those obtained by immunoblot analysis showing that synexin down-regulation resulted in down-regulation of intracellular galectin-3 levels (Fig. 6). Following apoptotic stimuli, galectin-3 was localized to the perinuclear membranes in BT549Gal-3 cells (Fig. 4*D*), whereas it remained evenly distributed in cells transfected with antisense oligonucleotides (Fig. 7*B*). Redistribution of galectin-3 proteins was consistently shown in BT549 cells into which antisense oligonucleotides were introduced at low efficiency (Fig. 7, *B* and *D*, *insets*). These results indicate that synexin expression is critical for intracellular galectin-3 expression and redistribution during apoptosis. We then tested whether disruption of galectin-3 translocation by down-regulation of synexin has effects on the anti-apoptotic activity of galectin-3. We measured staurosporine-induced DEVDase activity in BT549Gal-3 cells with or without synexin down-regulation (Fig. 7*E*). The basal levels of DEVDase activity in BT549Gal-3 cells were not significantly altered by synexin down-regulation using antisense oligonucleotides. However, DEVDase activity increased >3-fold following staurosporine treatment when synexin expression was down-regulated. This increase was similar to the level of DEVDase activation in BT549neo cells following the same treatment (Fig. 3*B*), showing that synexin down-regulation abolishes the ability of galectin-3 to down-regulate caspase activity.

(*SC+EF*), or with antisense oligonucleotides with Effectene (*AS+EF*). After treatment with 0 or 0.5 μM staurosporine for 150 min, DEVDase activities were measured and normalized per microgram of protein and are presented as relative fluorescence units (*RFU*). Three independent experiments were performed, and the *error bars* represent the mean ± S.D. of triplicates.

DISCUSSION

Galectins are a family of evolutionarily conserved animal lectins. During the past decade, efforts have been made to dissect the multiple functions of galectins. Recent studies including ours revealed that some members of the galectin family of proteins are novel regulators of apoptosis (14–16, 41–43). The galectin-7 gene is an early transcriptional target of the tumor suppressor gene product p53 following genotoxic stresses such as UV irradiation, and galectin-7 overexpression enhances keratinocyte apoptosis (44, 45). The extracellular galectin-1 and -9 proteins induce apoptosis in thymocytes or activated T-cells (41, 42). In contrast, our studies (15, 16) and others (14) indicate that intracellular, but not extracellular, galectin-3 inhibits apoptosis. When the recombinant galectin-3 proteins were supplemented in the medium at a concentration of up to 2 μg/ml, they failed to protect BT549 cells against apoptosis. Similarly, the conditioned medium containing galectin-3 secreted by galectin-3-overexpressing cells (BT549Gal-3) failed to down-regulate apoptotic events in BT549 cells, suggesting that extracellular galectin-3 lacks anti-apoptotic activity (data not shown). This study consistently indicates that cytoplasmic galectin-3 regulates epithelial cell apoptosis. In conjunction with recent clinical studies showing that cytoplasmic galectin-3 correlates with tumor progression (12, 13), this study may provide an explanation for the oncogenic activity of cytoplasmic galectin-3 in human carcinoma cells.

Following apoptotic stimuli, galectin-3 is enriched in the intracellular membrane including the mitochondria, a key regulation site for apoptosis induced by a variety of stimuli. Galectin-3, a non-Bcl-2 family member, effectively protects mitochondrial integrity and down-regulates the caspase cascade following intrinsic apoptotic signals. Many Bcl-2 family proteins reside in the mitochondrial outer membrane, endoplasmic reticulum, and nuclear envelope through their C-terminal membrane anchor domains (reviewed in Ref. 46). The Bcl-2 family proteins in the mitochondrial membrane are thought to interact with the permeability transition pore complex and to regulate the opening of the conductance channel. Unlike Bcl-2 family members, galectin-3 does not have a membrane anchor domain. Structurally, galectin-3 is composed of two distinct domains: an N-terminal domain containing proline- and glycine-rich sequences and a globular C-terminal domain containing the carbohydrate recognition site (5). Galectin-3 contains four amino acid residues (NWGR) that are conserved in the Bcl-2 homology domain 1 (BH1) of the Bcl-2 family. This motif is critical for Bcl-2 anti-apoptotic activity and its interaction with other Bcl-2 family members (47). Similar to the Bcl-2 protein, substitution of Gly^{182} with Ala in the NWGR motif of galectin-3 abrogates its anti-apoptotic function (15, 16). The galectin-3 protection of mitochondrial integrity may result from its ability to interact with Bcl-2 family members, as previously suggested (14), or galectin-3 may directly interact with the mitochondrial permeability transition pore complex and regulate its opening. This study shows that, although its molecular action is still unclear, galectin-3 exerts its anti-apoptotic activity at the perinuclear mitochondrial membranes. Caspases in cell-free extracts prepared from BT549Gal-3 cells can be effectively activated by addition of cytochrome *c*. This suggests that once significant amounts of cytochrome *c* are released from the mitochondria, galectin-3 fails to inhibits apoptotic events. Thus, galectin-3 inhibition of cytochrome *c* release from the mitochondria is critical for apoptosis inhibition.

Synexin (annexin VII), a 51-kDa member of the annexin family of proteins, can bind to lipid membranes (38, 48). Although the exact physiological function of synexin remains unknown, it has been proposed to act as a Ca^{2+} channel and as a Ca^{2+}-activated GTPase, thus regulating intracellular vesicle fusion and membrane trafficking (38, 48–50). Galectin-3 contains no signal sequence for its subcellular localization and is present in the nucleus and cytoplasm. It is also secreted by a mechanism independent of the endoplasmic reticulum-Golgi secretory vesicle pathway. It has been proposed that galectin-3 is targeted to the plasma membrane through an unknown mechanism and secreted through vesicular budding, followed by release from the vesicle. Down-regulation of synexin prevents galectin-3 translocation to the perinuclear membranes and increases galectin-3 secretion. This shows that synexin does not direct galectin-3 exocytosis, but is required for intracellular translocation of galectin-3. Thus, it appears that galectin-3 secretion and intracellular translocation employ two different pathways and that disruption of intracellular galectin-3 localization indirectly promotes the galectin-3 secretion pathway, suggesting a cross-talk between these pathways. Synexin regulates vesicle fusion in a Ca^{2+}-dependent manner (38, 48, 49). Interestingly, galectin-3 secretion was shown to be markedly stimulated by the calcium ionophore A23187, which alters homeostasis (51), suggesting that Ca^{2+} may be a messenger for the cross-talk between the galectin-3 transport pathways.

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Appendix 2

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ORIGINAL PAPERS

Galectin-3 enhances cyclin D_1 promoter activity through SP1 and a cAMP-responsive element in human breast epithelial cells

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Galectin-3 is a multifunctional carbohydrate-binding protein found in the nucleus, cytoplasm and the extracellular milieu. Nuclear galectin-3 expression is associated with cell proliferation, and its role in pre-mRNA splicing has been suggested. In this report, we investigated the role of galectin-3 on cyclin D_1 gene expression, a critical inducer of the cell cycle and a potential oncogene in human cancer. We found that galectin-3 induces cyclin D_1 promoter activity in human breast epithelial cells independent of cell adhesion through multiple cis-elements, including the SP1 and CRE sites. We present evidence that galectin-3 induction of the cyclin D_1 promoter may result from enhancement/stabilization of nuclear protein-DNA complex formation at the CRE site of the cyclin D_1 promoter. We also show that galectin-3 co-operates with, but does not depend on, pRb for cyclin D_1 promoter activation. The present study reveals a growth promoting activity of galectin-3 through cyclin D_1 induction, and suggests a novel function of nuclear galectin-3 in the regulation of gene transcription.
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① **Keywords:** ????

Introduction

Galectin-3 is a member of the β-galactosidase-binding gene family found abundantly in epithelial and immune cells. Galectin-3 is often highly expressed in human tumor cells and modulates a variety of cellular processes (Bresalier *et al.*, 1997; Konstantinov *et al.*, 1996; Lotan *et al.*, 1994; Nangia-Makker *et al.*, 1998; Raz *et al.*, 1989). Extracellular galectin-3 mediates cell migration, cell adhesion and cell–cell interactions, while intracellular galectin-3 regulates apoptosis (Akahani *et al.*, 1997; Kim *et al.*, 1999; Le Marer and Hughes, 1996; Sato and Hughes, 1992; Yang *et al.*, 1996). Galectin-3 is also found in the nucleus as a nuclear matrix protein and involved in pre-mRNA splicing (Dagher *et al.*, 1995; Wang *et al.*, 1995). We have previously shown that galectin-3 overexpression causes changes in expression levels of cell cycle regulators, including cyclin D_1 (Kim *et al.*, 1999), suggesting a role for galectin-3 in the regulation of gene expression. Structurally, galectin-3 is composed of two distinct domains: an N-terminal domain containing proline and glycine rich sequences and a globular C-terminal domain containing a carbohydrate recognition site (Raz *et al.*, 1989). Galectin-3 contains four amino acid residues (NWGR) that are conserved in the BH1 domain of the bcl-2 gene family. Substitution of the Gly^{182} residue with Ala in the NWGR motif of galectin-3 abrogates its anti-apoptotic function and its ability to modulate expression levels of cell cycle regulators (Akahani *et al.*, 1997; Kim *et al.*, 1999).

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 B-cell lymphomas, squamous cell carcinomas of the head, neck and esophagus, and breast cancer (Motokura and Arnold, 1993). Cyclin D_1 forms a complex with cyclin-dependent kinases (CDK4/6) and regulates progression of the early to mid G_1 phase of the cell cycle (Pines, 1995; Sherr, 1994). Studies have suggested that cyclin D_1 expression is the rate limiting step for G_1 progression (Lukas *et al.*, 1995; Resnitzky and Reed, 1995). Growth factors activate signal transduction pathways, including the ras-raf-p42/p44MAPK cascade, phospholipid turnover, and $p60^{src}$ pathways, resulting in activation of the cyclin D_1 promoter (Albanese *et al.*, 1995; Lavoie *et al.*, 1996; Lee *et al.*, 1999; Watanabe *et al.*, 1996a,b). The cyclin D_1 promoter contains multiple binding sites for transcription factors, including AP-1, signal transducers and activators of transcription (STAT), nuclear factor kappa B (NF-κB), cAMP-responsive element binding protein (CREB)/activating transcription factor (AFT), E2F, and SP1 (Pestell *et al.*, 1999). Growth factor activation of the cyclin D_1 promoter is mediated largely by these elements (Guttridge *et al.*, 1999; Hinz *et al.*, 1999; Joyce *et al.*, 1999; Matsumura *et al.*, 1999; Nagata *et al.*, 2001; Watanabe *et al.*, 1996a). However,

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studies demonstrated that growth factor signaling alone is not sufficient, and cell adhesion signaling is required for cyclin D_1 transcription (Le Gall *et al.*, 1998; Zhu *et al.*, 1996). Cell adhesion-activated cyclin D_1 promoter appears to occur through focal adhesion kinase (Zhao *et al.*, 1998) and integrin-linked kinase (ILK) involving CREB-dependent pathway (D'Amico *et al.*, 2000). In addition, the tumor suppressor protein pRB (retinoblastoma protein) was shown to be involved in cyclin D_1 regulation, through sequestration of E2F, an inhibitor of cyclin D_1 promoter activity, or by interaction with other transcription factors such as SP1, Ets, and Myc proteins (Albanese *et al.*, 1995; Kim *et al.*, 1992; Philipp *et al.*, 1994; Watanabe *et al.*, 1998).

In the present study, we investigated the role of galectin-3 in the regulation of cyclin D_1 promoter activity. Here, we report that galectin-3 induces cyclin D_1 promoter activity independent of cell adhesion. Galectin-3 induction of cyclin D_1 promoter is mediated by multiple cis-elements including SP1 and CREB binding sites. Galectin-3 co-operates with, but does not depend on, pRB for cyclin D_1 promoter activation. We also present evidence that galectin-3 induction of cyclin D_1 promoter activity may result from enhancement/stabilization of nuclear protein-DNA complex formation at the CRE site of the cyclin D_1 promoter.

Results

Galectin-3 activates cyclin D_1 promoter activity

Immunoblot analysis (Figure 1a) showed that cyclin D_1 protein expression is significantly upregulated in galectin-3 overexpressing BT549 cells (BT549-Galwt), but not in mutant galectin-3 overexpressing cells (BT549-Galm). To determine whether galectin-3 overexpression results in upregulation of cyclin D_1 expression at the transcriptional level, a luciferase reporter gene under the control of 964 bp cyclin D_1 promoter (−964CD1LUC) was introduced into control vector transfected (BT549neo), BT549-Galwt, and BT549-Galm cells. Cyclin D_1 promoter activity was ~4-fold higher in BT549-Galwt cells compared to the level in BT549neo cells, whereas the cyclin D_1 promoter activity was comparable between BT549-Galm and BT549neo cells (Figure 1b). These results showed that wild-type galectin-3 specifically upregulates cyclin D_1 promoter activity.

To exclude the possibility that increased cyclin D_1 promoter activity in BT549-Galwt cells results from clonal selection and/or long term cell culture with stable galectin-3 expression, we examined the role of galectin-3 in cyclin D_1 promoter activation by transient transfection into BT549 and MCF-7, both human breast carcinoma cell lines. Cyclin D_1 promoter activity was enhanced by galectin-3 in a dose-dependent manner; transient transfection of 1.5 μg galectin-3 expression plasmid enhanced cyclin D_1 promoter activity ~10-fold in both BT549 (Figure 2a) and MCF-7 (Figure 2b) cells. As a control experiment, an expression vector containing galectin-3 cDNA in a reverse orientation was introduced into BT549 cells. Galectin-3 expression (sense) vector, but not an antisense construct, induced cyclin D_1 promoter activity (Figure 2c). As an additional control experiment, the effects of galectin-3 expression on β-actin or luciferase reporter gene expression under thymidine kinase promoter with two copies of palindrome thyroid hormone receptor response element TRE (pTK-Pal-Luc, a gift from Dr JL Jameson at Northwestern University, Chicago, IL, USA) were also examined. There was no increase in β-actin expression or luciferase activity using the pTK-Pal-Luc construct when co-transfected with galectin-3 expression plasmid

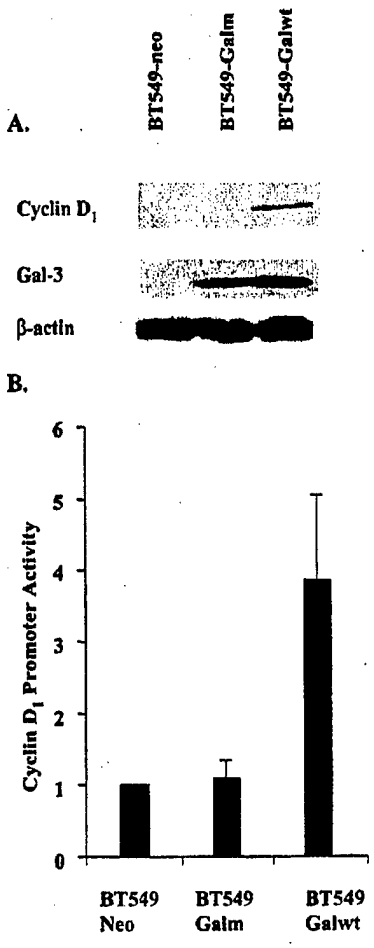

Figure 1 Galectin-3 induces cyclin D_1 expression through cyclin D_1 promoter activation in BT549 cells. (a) Lysates (10 μg/lane) of BT549neo, BT549-Galm, and BT549-Galwt were subjected to immunoblot analysis with an anti-cyclin D_1, anti-galectin-3, or anti β-actin antibody. Detection of the antigen was performed using ECL. (b) BT549neo, BT549-Galm, and BT549-Galwt cells were co-transfected with 3 μg of luciferase reporter construct (−964CD1LUC) and 0.15 μg of the β-galactosidase expression plasmid (pMDV-lacZ) using FuGENE 6 reagent. Cells were harvested at 36 h after transfection and the luciferase activity in 1 μg of cell lysate was normalized to β-galactosidase activity. The luciferase activity in BT549neo cells was arbitrarily given as 1. The data represent the average of three separate transfection experiments. Error bars represent s.d. of the mean

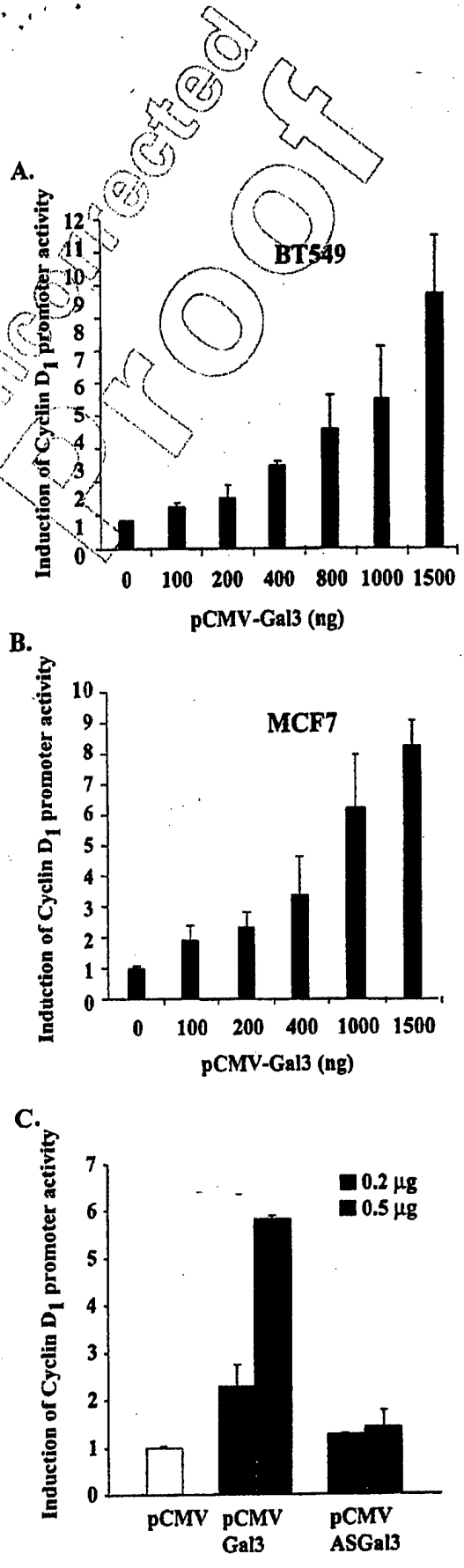

Figure 2 Transient transfection confirms galectin-3 activation of cyclin D_1 promoter in BT549 and MCF7 cells. Six μg of −964CD1LUC plasmid, 0.2 μg of pMDV-lacZ and increasing amounts of galectin-3 expression vector were co-transfected into BT549 (a) or MCF7 (b) cells. The plasmid pCRII (purchased from Invitrogen) 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 for transfection efficiencies. The luciferase activity in cells transfected without galectin-3 expression plasmid was arbitrarily given as 1. Error bars represent s.d. of the mean of triplicate samples. (c) BT549 cells were transfected with 6 μg of −964CD1LUC plasmid, 0.2 μg of pMDV-lacZ, and increasing amounts of sense or anti-sense galectin-3 expression vector. The luciferase activity in 1 μg of cell lysate was normalized to β-galactosidase activity. The luciferase activity in cells transfected with control vector without galectin-3 cDNA insert was arbitrarily given as 1

(data not shown). These control experiments further suggest that cyclin D_1 promoter activation was galectin-3 specific.

Galectin-3 activates cyclin D_1 promoter in the absence of cell adhesion

It was previously shown that cell adhesion signaling is a pre-requisite for growth factor-induced cyclin D_1 expression and that cyclin D_1 gene transcription is downregulated following the loss of cell adhesion (Le Gall *et al*., 1998; Zhu *et al*., 1996). Thus, we questioned whether galectin-3-induced cyclin D_1 expression is cell adhesion signaling-dependent. Immunoblot analysis showed that the cyclin D_1 protein level was significantly higher in suspension cultures of BT549-Galwt cells compared to those in suspension cultures of BT549-Galm and BT549neo cells (Figure 3a). Similarly, cyclin D_1 promoter activity was ~5-fold higher in suspension cultures of BT549-Galwt cells (Figure 3b) than those of BT549-Galm or BT549neo cells, demonstrating that cyclin D_1 promoter activation by galectin-3 is independent of cell adhesion. We further confirmed cell adhesion-independent galectin-3-induction of cyclin D_1 promoter activity by transient transfection studies. The cyclin D_1 promoter activity was reduced ~75% in suspension culture (using polyHEMA-coated dishes which prevents cell adhesion) compared to the monolayer culture of control MCF10A cells, a spontaneously immortalized 'normal' human breast epithelial cell line. However, less than a 30% decrease in cyclin D_1 promoter activity was detected in MCF10A cells when galectin-3 is transiently transfected (Table 1). In suspension cultures of MCF10A cells, galectin-3 enhanced cyclin D_1 promoter activity ~3-fold (0.23 *vs* 0.71). Galectin-3 also effectively induced cyclin D_1 promoter activity in suspension cultures of breast carcinoma MCF-7 (1.23 *vs* 9.45) and BT549 (0.86 *vs* 3.05) cells. It should be noted that galectin-3 induction of cyclin D_1 promoter activity in breast carcinoma cells was even greater in the absence of cell adhesion than in the presence of cell adhesion, suggesting that additional genetic changes in cancer cells may synergize with galectin-3 to further enhance cyclin D_1 promoter activity in the absence of

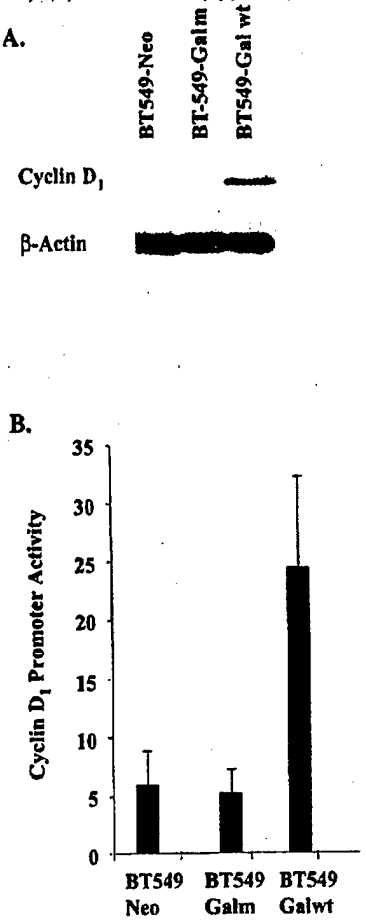

Figure 3 Galectin-3 induces cyclin D_1 expression in the absence of cell adhesion. (a) Lysates (10 μg/lane) of suspension cultures of BT549neo, BT549-Galm, and BT549-Galwt cells were subjected to immunoblot analysis with an anti-cyclin D_1 or anti-β-actin antibody. Detection of the antigen was performed using ECL. (b) BT549neo, BT549-Galm, and BT549-Galwt cells were co-transfected with 3 μg of luciferase reporter construct (−964CD1LUC) and 0.15 μg of the β-galactosidase expression plasmid (pMDV-lacZ) using FuGENE 6 reagent. Next day, cells were trypsinized and cultured in suspension for an additional 24 h before harvest for luciferase activity assay. The luciferase activity in 1 μg of cell lysate was normalized to β-galactosidase activity. The data represent the average of three separate transfection experiments. Error bars represent s.d. of the mean

Table 1

	Monolayer		*Suspension*	
	Control	*pCMV-Gal3*	*Control*	*pCMV-Gal3*
MCF10A	1	1.10±0.08	0.23±0.05	0.71±0.07
MCF7	1	5.19±2.26	1.23±0.15	9.45±0.07
BT549	1	2.65±0.77	0.86±0.09	3.05±0.49

cell adhesion signaling. Taken together, these results demonstrate that galectin-3 induces cyclin D_1 promoter activity regardless of cell adhesion in human breast epithelial cells.

Mutational analysis of the human cyclin D_1 promoter activity

To determine the cis-element(s) in the cyclin D_1 promoter region responsive to galectin-3, we transfected luciferase reporter constructs containing various lengths of the cyclin D_1 promoter (Figure 4) into BT549neo, BT549-Galwt, and BT549-Galm cells. Promoter activity of −163CD1LUC was comparable to −1745CD1LUC, −1093CD1LUC or −964CD1LUC (data not shown). Further deletion of E2F binding sites (−141CD1LUC) or mutation in E2F binding site (−163E2F*CD1LUC) did not reduce the luciferase activity, as shown in Figure 4. When the SP1 sites were deleted (−66CD1LUC), the promoter activity decreased to less than half of −141CD1LUC activity in BT549-Galwt cells. However, −66CD1LUC activity was still significantly higher in BT549-Galwt cells than those in BT549neo and BT549-Galm cells. This suggests that galectin-3-induced cyclin D_1 promoter activity significantly depends on the SP1 sites, and that additional cis-element(s) downstream of SP1 also play a role in galectin-3 induction of cyclin D_1 promoter activity.

The cyclin D_1 promoter contains a CRE site downstream of the SP1 sites. CREB binding was recently shown to mediate integrin-linked kinase-induced cyclin D_1 promoter activity (D'Amico *et al.*, 2000), suggesting that the CRE site is critical for cell adhesion-mediated cyclin D_1 promoter activation. To examine the role of this CRE element for galectin-3 regulation of cyclin D_1 promoter, −35CD1LUC lacking CRE site was transfected into BT549 neo, BT549-Galwt and BT549-Galm cells. Further deletion of the CRE site in the cyclin D_1 promoter almost completely abolished cyclin D_1 promoter induction by galectin-3. These results showed that SP1 and CRE elements are largely responsible for galectin-3 induction of cyclin D_1 promoter activity.

Galectin-3 co-operates with pRb for cyclin D_1 promoter activation

Cyclin D_1-associated kinase complex phosphorylates and inactivates pRb (Goodrich *et al.*, 1991; Weinberg, 1995). Interestingly, although pRb is not a transcription factor, pRb expression was shown to activate cyclin D_1 promoter in murine fibroblast cell line NIH3T3 and the human trophoblast cell line JEG-3, suggesting a negative feedback regulation loop between pRb and cyclin D_1 (Watanabe *et al.*, 1998). Since our previous study revealed that stable overexpression of galectin-3 upregulates pRb expression in BT549 cells (Kim *et al.*, 1999), we asked if pRb is involved in galectin-3 regulation of cyclin D_1 in human breast epithelial cells. Transient transfection of 0.5 μg pRb expression vector (pCMV-Rb) alone induced cyclin D_1 promoter activity 2–4-fold (Figure 5). Similarly, transient transfection of 0.5 μg galectin-3 expression vector (pCMV-Gal3) enhanced cyclin D_1 promoter activity 3–4-fold without detectable pRb upregulation

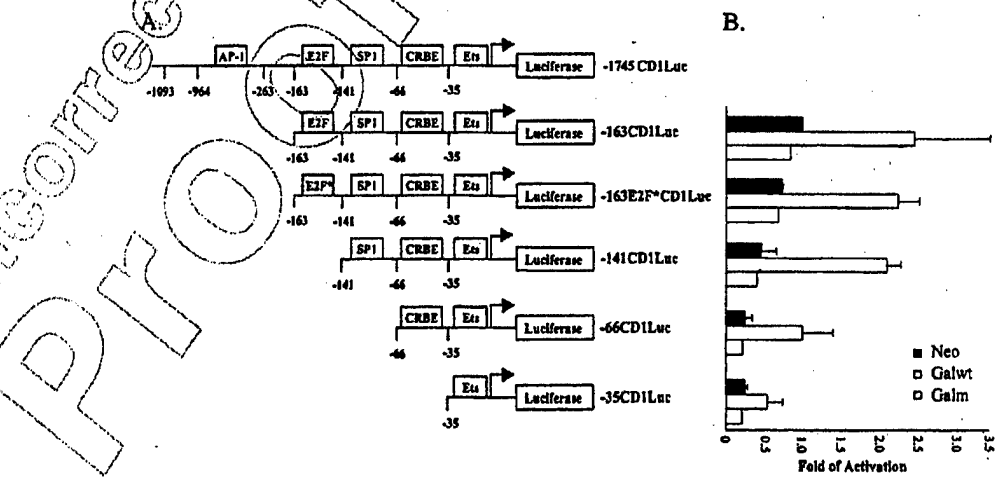

Figure 4 Galectin-3 induction of cyclin D_1 promoter involves SP1 and CREB pathways. (a) Schematic representation of the luciferase reporter constructs containing various lengths of the cyclin D_1 promoter. Diagrams depict locations of potential transcription factor binding sites in the cyclin D_1 promoter. (b) The luciferase reporter constructs were transfected into BT549-neo, BT549-Galwt, and BT549-Galm cells. Cells were harvested and analysed for luciferase activity at 48 h after transfection. The luciferase activity in 1 μg of cell lysate was normalized to β-galactosidase activity. The luciferase activity of -163CD1LUC1 in BT549neo cells was arbitrarily given as 1

(Of note, * in Figure 5 indicates nonspecific bands recognized by anti-Rb antibody). These results showed that pRb upregulates cyclin D_1 promoter activity in human breast epithelial BT549 cells similar to murine fibroblasts and human trophoblast cells. These results also showed that pRb is unlikely to be required for galectin-3 upregulation of cyclin D_1 promoter activity. When increasing amounts of pRb expression vector were transfected together with -964CD1LUC reporter construct and galectin-3 expression vector, the cyclin D_1 promoter activity further increased (Figure 5a). In a reciprocal experiment, when increasing amounts of galectin-3 expression vector were transfected together with -964CD1LUC reporter construct and pRb expression vector, the cyclin D_1 promoter activity also further increased (Figure 5b). The cyclin D_1 promoter induction by galectin-3 and pRb was additive, not synergistic. These results suggest that pRb and galectin-3 cooperate for cyclin D_1 promoter activation, but not necessarily depend on each other.

Galectin-3 enhances nuclear protein binding to the CRE site of the cyclin D_1 promoter

The present study showed that galectin-3 activates cyclin D_1 promoter even in the absence of cell adhesion signaling, possibly through SP1 and CRE sites. Since a recent study suggests that the CRE site mediates cell adhesion-regulated cyclin D_1 promoter activity (D'Amico *et al.*, 2000), we questioned whether galectin-3 enhances CRE-mediated cyclin D_1 promoter activation, which compensates for a decrease in cell adhesion signaling. To this end, we examined whether nuclear proteins from BT549-Galwt cells bind more effectively to the cyclin D_1 CRE site compared to nuclear proteins from BT549neo cells. Nuclear proteins formed two major complexes (band A and B) with a radiolabeled double-stranded oligonucleotide containing the cyclin D_1 CRE site in the presence of 100-fold molar excess of non-specific competitor poly(dI-dC) (Figure 6). Both complexes were competed out by 10-fold molar excess of unlabeled CRE oligonucleotides, indicating that these binding were CRE sequence specific. The density of these bands was significantly enhanced when nuclear extract of BT549-Galwt was used.

To determine the role of galectin-3 in nuclear protein binding to the CRE sequence, nuclear proteins were pre-incubated with anti-galectin-3 antibody prior to the binding reaction. The density of band B was drastically reduced by anti-galectin-3 antibody ($\sim$10-fold as quantified using Biorad Quantity One program), whereas incubation with preimmune serum had no detectable effect on the DNA-protein complex formation (Figure 6b). These results suggest that galectin-3 enhances transcription factor complex binding to the CRE site, and anti-galectin-3 antibody binding to galectin-3 disrupted nuclear protein/DNA complex formation (especially band B) at the CRE site. To further confirm this, we examined the effect of recombinant galectin-3 protein on nuclear protein binding activity at the CRE site. The purity of recombinant galectin-3 protein was assured by polyacrylamide-SDS gel electrophoresis/Coomassie blue staining and immunoblot analysis (data not shown). Recombinant galectin-3 protein alone had no DNA binding activity at the cyclin D_1 CRE oligonucleotide (data not shown). However, when the effect of recombinant galectin-3 protein on BT549 nuclear protein binding to the CRE site was tested, $\sim$60%

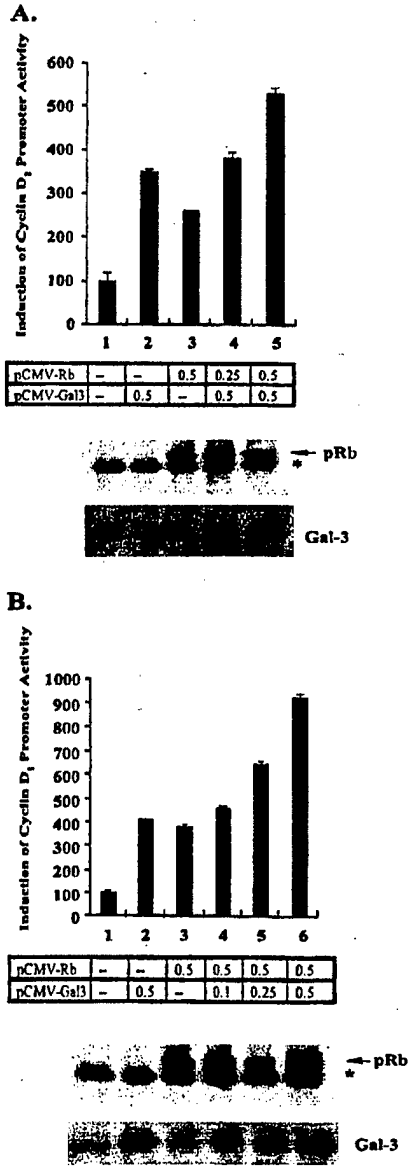

Figure 5 Galectin-3 co-operates with, but does not depend on, pRb for cyclin D_1 promoter activation. BT549 cells were transfected with 3 μg of −964CD1LUC, 0.15 μg of the β-pMDV-lacZ, and the indicated combination of pCMV-Gal3 and pCMV-Rb plasmids (μg). The luciferase activity in 1 μg of cell lysate was normalized to β-galactosidase activity. The luciferase activity without galectin-3 and pRb plasmid transfection was arbitrarily given as 100. The pRb and galectin-3 protein levels after transfections were detected by immunoblot analysis (lower panels). The asterisk indicates a nonspecific band recognized by anti-Rb antibody (Santa Cruz Biotechnology)

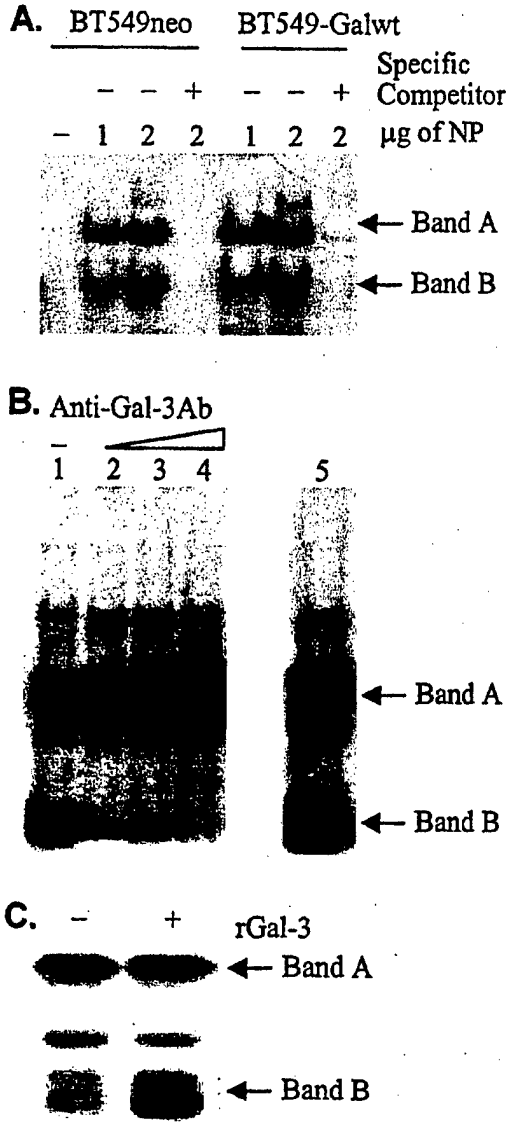

Figure 6 Galectin-3 enhances/stabilizes nuclear protein binding to the CRE site. (**a**) One or two μg of nuclear extracts prepared from BT549neo and BT549-Galwt cells were incubated with 0.2 ng of ^{32}P-dCTP-labeled double-stranded (ds) oligonucleotides containing the CREB binding site of the cyclin D_1 promoter in the presence of 100-fold molar excess of non-specific competitor poly(dI-dC), and subjected to mobility shift assay as described in Materials and methods. As a specific competitor, 10-fold molar excess of cold probe was added to reaction. Protein-DNA complexes were separated on a 5% non-denaturing polyacrylamide gel at 100 V for 2 h. Arrows indicate nuclear protein/CRE complexes (band A and B). (**b**) Equal amount of nuclear proteins prepared from BT549-Galwt cells were incubated with increase amounts of rat monoclonal anti-galectin-3 (Lanes 2–4) or pre-immune serum (Lane 5). Complexes were resolved on a 5% polyacrylamide gel at 100 V for 2.5 h. (**c**) The ^{32}P-labeled oligonucleotides containing the CRE site were incubated with 2 μg of nuclear proteins from BT549 cells in the presence or absence of 1 μg of purified recombinant galectin-3 proteins (rGal-3). The complexes were resolved in 6% polyacrylamide gel at 100 V for 2.5 h. Arrows indicate the band A and B complexes. Of note, the additional band migrating between the band A and B is a non-specific band whose intensity and existence varied among experiments

enhancement of band B formation (as quantified using Biorad Quantity One program) was observed (Figure 6c), further suggesting a role for galectin-3 in protein/DNA complex formation at the CRE site. We also examined whether galectin-3 has any effect on nuclear protein binding to the SP1 site. Although mutational

analysis of the human cyclin D_1 promoter clearly showed significance of the SP1 site for galectin-3 induction of cyclin D1 promoter activity (Figure 4), we found no detectable difference in protein-DNA complex formation using oligonucleotides containing SP1 sites only (data not shown). Taken together, the present study suggests that nuclear galectin-3 may enhance/stabilize transcription factor binding to the CRE site in the cyclin D1 promoter, which then promotes the transactivation functions of transcription factors such as CREB and SP1. This notion is consistent with the previous reports that galectin-3 is a nuclear matrix protein (Wang *et al.*, 1995) and its expression in the nucleus is associated with cell proliferation (Openo *et al.*, 2000). We also confirmed galectin-3 expression in the nucleus of BT549-Galwt cells by immunofluorescence study (Figure 7). Galectin-3 protein was evenly detected in the nucleus and the cytoplasm in logarithmically growing BT549-Galwt cell (Figure 7a). To confirm that galectin-3 is expressed within the nucleus, galectin-3 stained cells were mounted with medium containing DAPI (4′,6 diamidino-2-phenylindole, Vector Laboratory, CA, USA) which produces a blue fluorescence when bound to DNA (Figure 7b). Confocal microscopic analysis from the same plane of focus revealed that galectin-3 and DNA staining co-localize inside the nucleus (Figure 7c). In contrast, no detectable galectin-3 expression was found in the nucleus of quiescent BT549-Galwt cells cultured in serum-free medium for 48 h (Figure 7d), suggesting a correlation between nuclear galectin-3 expression and cyclin D_1 expression during cell proliferation.

Discussion

The present study showed that galectin-3 upregulates cyclin D_1 promoter activity, a critical inducer of the cell cycle and a potential oncogene in human cancer (Lukas *et al.*, 1995; Motokura and Arnold, 1993; Pines, 1995; Resnitzky and Reed, 1995; Sherr, 1994), revealing a part of growth promoting/oncogenic action of galectin-3. Although galectin-3 does not directly bind to the cis-element of the cyclin D_1 promoter, the present study suggests a role for nuclear galectin-3 in the regulation of cyclin D_1 promoter through enhancement/stabilization of CRE-associated complex formation. This notion is consistent with recent reports that the cellular expression of galectin-3 is proliferation-dependent (Kadrofske *et al.*, 1998; Openo *et al.*, 2000). While the galectin-3 protein was predominantly cytoplasmic in senescent human fibroblast cells, it was found in both the nucleus and the cytoplasm of proliferating cells (Openo *et al.*, 2000). Similarly, we showed that the galectin-3 protein is found in the nucleus of proliferating, but not quiescent, human breast epithelial cells.

Vascular endothelial growth factor-induced cyclin D_1 promoter activity was shown to be regulated by a dual system. One, mediated by SP1, is inducible in the G_1 phase, while the other, mediated by CREB, is constitutively active when cultured in monolayer (Nagata *et al.*, 2001). The integrin-linked kinase was shown to activate the CRE-dependent pathway for cyclin D_1 transcription. Thus, it appears that CREB mediates cell adhesion-dependent cyclin D_1 transcription, a pre-requisite for cyclin D_1 transcription, and SP1 mediates growth factor-induced cyclin D_1 transcription. Interestingly, the present study showed that galectin-3 induction of cyclin D_1 promoter activity also involves both SP1 and CREB pathways. The ability of galectin-3 to enhance both the SP1 and CREB pathways may explain how galectin-3 effectively induces cyclin D_1 expression in the absence of cell adhesion.

At present, it is unclear how galectin-3 co-operates with pRb for cyclin D_1 promoter activation. pRB induction of cyclin D_1 was suggested to occur through sequestration of E2F, an inhibitor of cyclin D_1 promoter activity, or by interaction with other transcription factors such as SP1 (Watanabe *et al.*, 1998). Galectin-3 effectively induced cyclin D_1 promoter activity for both −163CD1LUC and

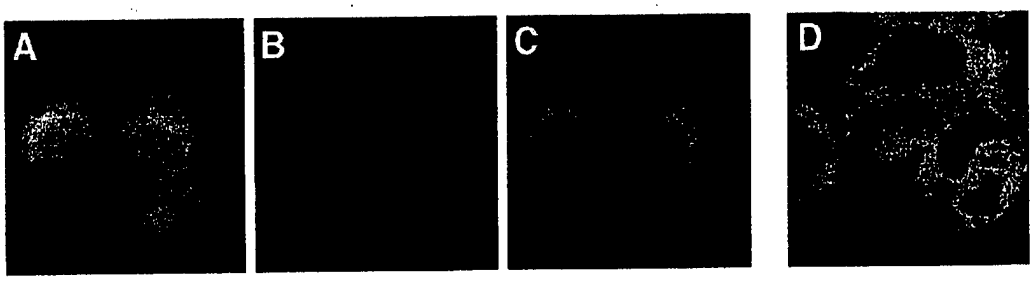

Figure 7 Galectin-3 is expressed in the nucleus of proliferating, but not quiescent, human breast epithelial cells. BT549-Galwt cells (a–c) were cultured on a cover slip with serum-containing medium for 48 h, washed with PBS three times, fixed with 3.7% formaldehyde in PBS for 15 min, washed with PBS-S (PBS containing 0.1% saponin), and then stained with rat anti-galectin-3 antibody followed by incubation with FITC-conjugated secondary antibodies and with anti-fade mounting medium containing 1.5 μg/ml DAPI (Vector Laboratory, CA, USA). Galectin-3 protein was shown in green (a), the nuclei stained with DAPI shown in blue (b), and nuclear galectin-3 shown in light blue in the confocal mode from the same plane of focus with a Zeiss LSM microscope (c). (d) BT549-Galwt cells were cultured on cover slip with serum free medium for 48 h, and stained with rat anti-galectin-3 antibody as described above

−163FCD1LUC which contains a mutation in the E2F site that abolished E2F binding (Figure 4). Thus, it is unlikely that galectin-3 activation of cyclin D_1 involves pRB-E2F pathway. Galectin-3 together with pRb may stabilize transcription factor complexes, including CREB, SP1 and others, resulting in transactivation of their transcription factor function.

The present study suggests a novel function of nuclear galectin-3 in the regulation of gene transcription. Galectin-3, a nuclear matrix protein, appears to enhance/stabilize transcription factor binding to the cis-elements of the cyclin D_1 promoter, including CRE and possibly SP1 sites. Since CRE and SP1 sites are often found in the promoter region of many genes, galectin-3 may also modulate transcription of other genes. In fact, our previous study showed that galectin-3 overexpression results in changes in expression levels of other cell cycle regulators. Galectin-3 upregulates expression of cyclin A, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, whereas it downregulates cyclin E expression (Kim *et al*., 1999). These changes may result from galectin-3 regulation of transcription factor complexes (enhancer and/or silencer complexes). It is equally possible that many of these effects are indirect. Galectin-3 mediated cyclin D_1 upregulation may have subsequent effects on expression levels of other cell cycle regulators. It was previously shown that cyclin D_1 overexpression alone is sufficient to trigger cell cycle transition involving changes in expression levels of other cell cycle regulators (Bienvenu *et al*., 2001; Latella *et al*., 2001). At present, the generality and specificity of galectin-3 regulation of gene transcription and its direct and indirect effects remain to be investigated.

We and others have previously shown that galectin-3 inhibits apoptosis induced by a variety of stimuli including staurosporine, cisplatin, genistein, and anoikis (apoptosis induced by the loss of cell adhesion) (Akahani *et al*., 1997; Kim *et al*., 1999; Lin *et al*., 2000; Matarrese *et al*., 2000; Matarresea *et al*., 2000; Yang *et al*., 1996). Galectin-3 inhibition of anoikis involves cell cycle arrest at an anoikis-insensitive point (late G_1) through modulation of gene expression and the activities of cell cycle regulators (Kim *et al*., 1999). Galectin-3 translocation to the nucleus and its phosphorylation are required for induction of cyclin D_1 expression and inhibition of anoikis (Yoshii *et al*., 2002). However, galectin-3 regulation of cyclin D_1 in the nucleus is unlikely to be a common mechanism for apoptosis regulation. Following a variety of apoptotic stimuli, galectin-3 translocates to the perinuclear membrane and prevents mitochondrial damage and cytochrome *c* release, thereby effectively protecting cells against apoptosis induced by agents working through different apoptosis initiation pathways (Yu *et al*., 2002). Likewise, in quiescent cells cultured in serum-free medium, galectin-3 translocates to the perinuclear membrane and inhibits apoptosis (Figure 7d and (Yu *et al*., 2002)). Thus, galectin-3 induction of cyclin D_1 is likely to be associated with its growth promoting activity and have effects on only a subset of apoptotic processes where cell cycle regulation is critical (e.g., anoikis).

Materials and methods

Cell culture

The human breast carcinoma cell line BT549 was obtained from Dr EW Thompson (Georgetown University Medical Center). Establishment of neo-resistant control vector transfected BT549 (BT549neo), galectin-3 transfected BT549 (BT549-Galwt), and mutant galectin-3 expressing BT549 (BT549-Galm) was previously described (Akahani *et al*., 1997; Kim *et al*., 1999). The mutant galectin-3 protein was generated by substitution of the Gly^{182} residue in the NWGR motif with Ala (Akahani *et al*., 1997; Kim *et al*., 1999). BT549 cells were cultured in RPMI1640 with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (50 units/ml). MCF7 cells, a human breast carcinoma cell line, were cultured in DMEM/F12 with 10% donor calf serum, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (50 units/ml). MCF10A cells, a spontaneously immortalized 'normal' breast epithelial cell line, were cultured in DMEM/F12 medium supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 20 ng/ml EGF, 0.1 μg/ml cholera enterotoxin, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 0.5 μg/ml fungizone in a 95% air and 5% CO_2 incubator at 37°C.

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. Suspension culture was maintained in polyHEMA-coated 100-mm dishes in a 95% air and 5% CO_2 incubator.

Plasmid and transient transfection

The reporter constructs of human cyclin D_1 promoter −1745CD1LUC, −1093CD1LUC, −964CD1LUC, −163CD1LUC, −163E2FCD1LUC, and −66CD1LUC were previously described (Albanese *et al*., 1995; Watanabe *et al*., 1996a, 1998). The −141CD1LUC was constructed by partial digestion of −163CD1LUC with NarI and Klenow treatment, followed by SmaI digestion and self-ligation. The −35CD1LUC construct was created from −163CD1LUC by PCR using primers CCTTATGCAGTTGCTCTCCAGCGGTTCA and GGACTACCCGGGAGTTTTGTTGAAGTTGCA. Cells were transfected with 6 μg cyclin D_1 reporter plasmid and 0.2 μg β-galactosidase expression plasmid pMDV-lacZ in the presence or absence of increasing amounts of galectin-3 expression plasmid pCMV-Gal3 (Akahani *et al*., 1997) or pRb expression plasmid pCMV-RB (a gift from Dr Y-HP Lee at the University of Texas Health Science Center at San Antonio). PCRII plasmid DNA (Invitrogen, CA) was used to adjust equal amounts of DNA for each transfection. Transfection was performed using FuGENE 6 reagent (Roche Molecular Biochemicals, Germany) according to manufacturer's protocol. The transfection efficiency was normalized by β-galactosidase activity. To measure cyclin D_1 promoter activity in the absence of cell adhesion, cells were washed with PBS and trypsinized 16–24 h after transient 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, WI, USA) and Galacto-Light kit (Tropix Inc., CA, USA) 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, IL, USA). The data are representative of at least three independent experiments.

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 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-galectin-3, anti-cyclin D_1 (Calbiochem, CA, USA), or anti-pRb (Santa Cruz Biotech., CA, USA) antibody, and with the appropriate HRP-conjugated secondary antibodies. The antigen was detected using the ECL detection system according to the manufacturer's instruction (Pierce, IL, USA).

Nuclear extract preparation

Crude nuclear extracts were prepared as described (Lin *et al.*, 1998) with slight modification. Cells were washed with cold phosphate buffer and resuspended in 5 volumes of cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF). The cells were allowed to swell on ice and lysed by rapid extrusion through a $26\frac{1}{2}$ G needle. Samples were centrifuged and the pelleted nuclei were extracted in cold buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF, 0.5 mM DTT, 5 μg/ml leupeptin). Nuclear extracts were aliquoted and stored at −70°C until use.

Electrophoretic mobility shift assays

Double-stranded (ds) oligonucleotides containing the CREB binding site of the cyclin D_1 promoter were prepared by annealing two synthetic complementary oligomers (5′-AACA-ACAGTAACGTCACACGGACT-3′ and 3′-TTGTTGTCA-TTGCAGTGTGCCTGATGT-5′) in annealing buffer (0.5 M NaCl, 0.5 M Na_2HPO_4 pH 8.0) at 95°C for 5 min, 65°C for 10 min and 4°C for 2 h. The oligomers were labeled with [α-^{32}P]dCTP by Klenow fragment of DNA polymerase I. Binding reactions were carried out for 20 min at room temperature in 10 mM HEPES pH 7.9, 80 mM KCl, 1 mM EDTA, 1 mM DTT, 4% Ficoll. Five micrograms of nuclear proteins were incubated with ~0.2 ng of ds CRE oligonucleotides ($3-5\times10^4$ c.p.m.) in the presence of 100-fold molar excess of non-specific competitor poly(dI-dC). To confirm the specificity of protein binding to the CRE sequence, 5 μg of nuclear protein was incubated with 2 ng of unlabeled ds CRE oligonucleotides (10-fold molar excess of specific competitor) for 10 min prior to addition of ~0.2 ng radiolabeled CRE probe. To examine the galectin-3 involvement in nuclear protein binding at the CRE oligonucleotides, pre-immune rabbit serum, anti-galectin-3 antibody, or recombinant galectin-3 protein was incubated with nuclear proteins on ice for 30 min before binding reaction to the CRE probe. Protein-DNA complexes were resolved on 5 or 6% non-denaturing polyacrylamide gels (acrylamide : bisacrylamide 19 : 1) electrophoresed in 0.5×TBE (45 mM Tris-HCl, 45 mM Boric Acid, 0.5 mM EDTA). The gel was dried and autoradiographed.

Preparation of recombinant galectin-3 protein

LB medium containing 10 mM $MgCl_2$ and 100 μg/ml ampicillin was inoculated with an overnight culture of HMS-174 transformed with the plasmid containing the human galectin-3 cDNA insert. When the bacterial cells were grown to an optical density of 0.5, IPTG (1 mM) was added. Cells were then incubated for an additional 4 h and harvested by centrifugation at 1250 *g* at 4°C. The pellet was washed with PBS and suspended in 100 mM of ice-cold lysis buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris, pH 8, 0.241 unit/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.2 mM PMSF). The bacterial cells were disrupted by sonicator with multiple short bursts of maximum intensity (30s×4) on ice. The lysate was centrifuged at 40 000 *g* for 20 min and the supernatants were passed through an asialofetuin affinity column (100×22 mm inner diameter). The column was made by linking the asialofetuin to Affigel-15 (Bio-Rad, CA, USA) according to the manufacturer's protocol, and was well equilibrated in phosphate buffer (10 mM phosphate, 1 mM MgSO4, 0.2 mM PMSF, and 0.2% NaN_3, pH 7.2). The column was washed with 3–5 column volumes of phosphate buffer, and the bound protein was eluted with 0.2 M lactose. Eluted fractions were quantitated using BCA protein assay reagent (Pierce, IL, USA), analysed by SDS–PAGE and immunoblot analyses using an anti-galectin-3 antibody.

Abbreviations

The abbreviations used are: PBS, phosphate-buffered saline; CDK, cyclin-dependent kinase; CRE, cAMP-responsive element; CREB, cAMP-responsive element binding protein; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; IPTG, isopropylthio-β-D-galactoside.

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Galectin-3 mediates genistein-induced G_2/M arrest and inhibits apoptosis

Appendix 3

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Many recent studies have focused on potential chemopreventive activities of dietary genistein, a natural isoflavonoid compound found in soy products. Genistein has been implicated in anticancer activities, including differentiation, apoptosis, inhibition of cell growth and inhibition of angiogenesis. In previous studies, genistein was shown to induce apoptosis and cell cycle arrest at G_2/M in several cancer cell lines *in vitro*, which is associated with induction of $p21^{WAF1/CIP1}$, a universal inhibitor of cyclin-dependent kinases. At present, the molecular basis for diverse genistein-mediated cellular responses is largely unknown. In the present study, we investigated whether galectin-3, an anti-apoptotic gene product, regulates genistein-mediated cellular responses. We show that genistein effectively induces apoptosis without detectable cell cycle arrest in BT549, a human breast epithelial cell line which does not express galectin-3 at a detectable level. In galectin-3 transfected BT549 cells, genistein induced cell cycle arrest at the G_2/M phase without apoptosis induction. Interestingly, genistein induces $p21^{WAF1/CIP1}$ expression in galectin-3-expressing BT549 cells, but not in control BT549 cells undergoing apoptosis. Collectively, the results of the present study suggest that galectin-3, at least in part, is a critical determinant for genistein-mediated cell cycle arrest and apoptosis, and genistein induction of $p21^{WAF1/CIP1}$ is associated with cell cycle arrest, but not required for apoptosis induction.

Introduction

Epidemiological studies suggest that the soybean isoflavonoid genistein (4,5,7-trihydroxyisoflavone phytoestrogen) may decrease the incidence of certain types of cancer and reduce the age-adjusted death rates from breast cancer (1–3). Consistently, genistein has been shown to mediate cell differentiation (4), inhibit angiogenesis (5,6) and be cytotoxic in a wide variety of cancer cell lines *in vitro* (4,7–10). Experiments in mice also provide evidence for genistein-mediated cytotoxic/cytostatic activity (11). Several possible mechanisms for genistein's anticancer activity have been suggested. These include inhibition of protein tyrosine kinase and topoisomerase (7,8,12,13). In contrast to the anticancer activity of genistein, genistein enhances carcinogen-induced tumor formation in the mouse colon (14). Similarly, maternal exposure to genistein dose-dependently increases carcinogen-induced mammary tumor progression in female rat offspring, mimicking the effects of estrogen exposure (15). These studies suggest that genistein may enhance tumorigenic potential in an organ-specific and/or developmentally regulated manner. At present, the molecular basis for genistein-mediated diverse cellular effects is not known.

Genistein induces apoptosis and cell cycle arrest at G_2/M in a variety of cancer cell lines *in vitro*, which is associated with $p21^{WAF1/CIP1}$ induction, a universal inhibitor of cyclin-dependent kinases (4,10,16). Efforts have been made to determine the gene products critical for mediating genistein-induced cellular responses. Since early studies showed that genistein binds estrogen receptors (ERs) (32,33), the role of ERs on genistein-mediated effects in cancer cells was examined. No relationship was found between ER expression and genistein-induced cell cycle arrest or apoptosis (7). Similarly, inhibition of cell growth by genistein was shown to be independent of the tumor suppressor gene product p53 (16).

In the present study, we investigated whether galectin-3, an anti-apoptotic gene product, modulates genistein-mediated cellular responses. Galectins are a family of proteins that bind to galactose-containing ligands (17). Galectin-3, a 30 kDa member of the galectin family, is widely found in epithelial and immune cells, and is highly expressed in various human tumor cells, including breast cancer (18–22). Although the exact mechanism is unknown, galectin-3 expression is associated with neoplastic progression and metastatic potential (18–25). A recent study showed that galectin-3 inhibits anti-Fas antibody and staurosporine-induced apoptosis in T-lymphocytes (26). In accordance with this, we have demonstrated that galectin-3 inhibits apoptosis induced by cisplatin (27) and loss of cell adhesion (anoikis) (28), suggesting that the oncogenic activity of galectin-3 may involve apoptosis inhibition. Here, we report that genistein arrests cells at the G_2/M phase in galectin-3-expressing human breast epithelial cells without detectable apoptosis induction, while it rapidly induces apoptosis in the control cells without detectable cell cycle arrest. In addition, we provide evidence indicating that genistein-induced $p21^{WAF1/CIP1}$ is associated with cell cycle arrest at G_2/M but is not required for apoptosis.

Materials and methods

Cell culture and genistein treatment

The human breast cancer cell line BT549 was obtained from Dr E.W.Thompson, Vincent T.Lombardi Cancer Research Center, Georgetown University Medical Center, Washington, DC. Galectin-3-transfected BT549 cells (BT549-Galwt) were previously established by introducing an expression vector containing human galectin 3 cDNA into BT549 parental cells (27,28). BT549 cells transfected with the control vector conferring neomycin resistance are referred to as BT549neo. BT549 cells expressing a mutant galectin-3 in which Gly^{182}

Abbreviations: Ac-DEVD-amc, acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin; ER, estrogen receptor; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

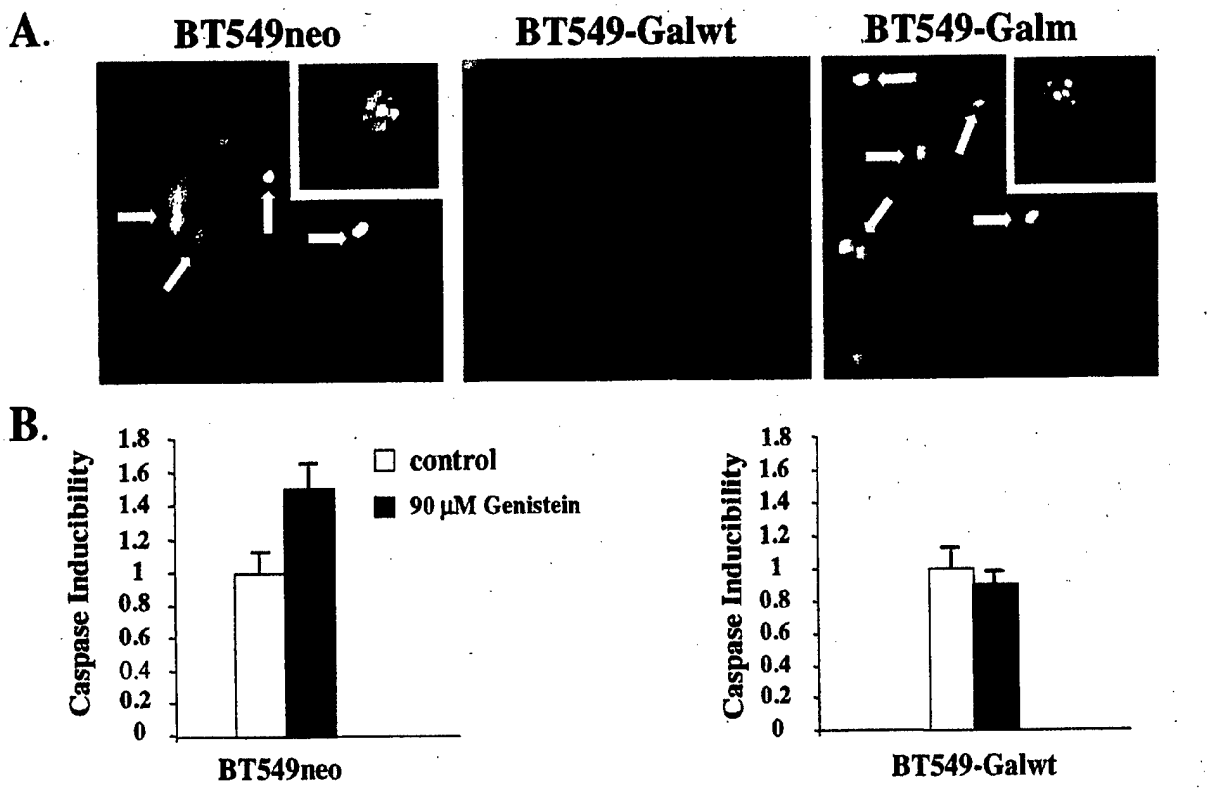

Fig. 2. Galectin-3 inhibits genistein-induced apoptosis. **(A)** BT549neo, BT549-Galwt and BT549-Galm cells were treated with 90 μM genistein for 48 h and analyzed for nuclear morphology using bisbenzimide staining. Arrows, apoptotic nuclei. Higher magnifications of the fragmented apoptotic nuclei of BT549neo and BT549-Galm cells are shown in the top, right corner. **(B)** BT549neo and BT549-Galwt cells were treated with 90 μM genistein for 48 h and DEVDase activity was assayed using Ac-DEVD-amc as a substrate. DEVDase activity was normalized per μg protein. The error bars represent standard deviation of the mean of triplicates.

Galwt cells increased to ~180% (Figure 1A). An additional 24 h exposure of cells to 90 μM genistein was cytostatic towards BT549-Galwt cells and was cytotoxic towards BT549neo cells (Figure 1B). This shows that galectin-3 expression protects BT549 cells against genistein-induced cytotoxicity.

Structurally, galectin-3 is composed of two distinct domains: an N-terminal domain containing proline- and glycine-rich sequences and a globular C-terminal domain containing the carbohydrate recognition site (18). Galectin-3 contains four amino acid residues, NWGR, that are conserved in the BH1 domain of the Bcl-2 family. This motif is critical for Bcl-2's anti-apoptotic activity (29). As in the Bcl-2 protein, substitution of the Gly^{182} residue with Ala in the NWGR motif of galectin-3 abrogates its anti-apoptotic function (27,28). To test whether the NWGR motif is required for galectin-3 inhibition of genistein-mediated cytotoxicity, we examined the effect of genistein on BT549-Galm cells, BT549 cells expressing mutant galectin-3 in which the Gly^{182} of the NWGR motif was substituted with Ala (27,28). As shown in Figure 1, mutant galectin-3 failed to protect BT549 cells against genistein-induced cytotoxicity, further substantiating the observation that the NWGR motif is critical for galectin-3's ability to prevent cell death.

Galectin-3 inhibits genistein-induced apoptosis and results in cell cycle arrest at G_2/M

To determine the mode of genistein-induced cell death, we examined nuclear morphology following genistein treatment. Genistein-treated BT549neo and BT549Galm cells underwent apoptotic changes, including chromosome condensation and fragmentation. In contrast, no significant change was observed in BT549-Galwt cells following genistein treatment (Figure 2A), suggesting that galectin-3 inhibits genistein-induced apoptosis. To confirm this, we measured caspase activity using the fluorogenic substrate Ac-DEVD-amc, a substrate for caspases 3, 6, 7, 8 and 10. Caspases are a family of cysteine proteases and their activation is regarded as the molecular instigator of apoptosis (30). Caspase activity in BT549neo and BT549-Galwt cells was determined by release of aminomethyl coumarin from the tetrapeptide substrate Ac-DEVD-amc. While genistein significantly induced DEVDase activity in BT549neo, there was no induction in BT549-Galwt cells. These results show that galectin-3 inhibits genistein-induced caspase activity and apoptosis in BT549 cells.

We have previously showed that galectin-3 inhibition of apoptosis is associated with its ability to arrest the cell cycle: cisplatin treatment or loss of cell anchorage induces cell cycle arrest at late G_1 in galectin-3-overexpressing cells, while it rapidly induces apoptosis in control cells (27,28). Next, we asked if the lack of apoptosis in BT549-Galwt cells following genistein treatment is also associated with galectin-3 involvement in cell cycle regulation. Genistein treatment of BT549neo cells resulted in a rapid decrease in the G_0/G_1 population (Figure 3A), and an increase in S phase and sub-G_1 (apoptotic) populations, suggesting that genistein triggers both cell cycle entry and apoptosis in these cells. However, genistein treatment reduced the G_2/M population of BT549neo cells. This suggests that these cells underwent apoptotic cell death before they reach the G_2/M phase. Genistein treatment of BT549-Galwt

cells also induced cell cycle entry, as detected by a reduction in G_0/G_1 population (Figure 3B). In contrast to BT549neo cells, no increase in the sub-G_1 population was detected in genistein-treated BT549-Galwt cells. Instead, with increased exposure time to genistein, BT549-Galwt cells accumulated at G_2/M, with reduced G_0/G_1 and S populations. These studies demonstrate that galectin-3 is a critical determinant for genistein-induced apoptosis and cell cycle arrest at G_2/M in BT549 cells.

$p21^{WAF1/CIP1}$ induction is associated with genistein-induced G_2/M arrest, but not with apoptosis

We previously reported that galectin-3 inhibition of anoikis involves cell cycle arrest at late G_1 through induction of cyclin D_1 (an early G_1 cyclin) and cyclin-dependent kinase inhibitors ($p21^{WAF1/CIP1}$ and $p27^{KIP1}$) (28). To understand the molecular basis for genistein-mediated cell cycle arrest at G_2/M in BT549-Galwt cells, the effect of galectin-3 expression on gene expression of cell cycle regulators was examined. Galectin-3 expression resulted in a basal level increase in cyclin D_1, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ as shown in Figure 4A. Genistein treatment further enhanced expression of cyclin D_1 in BT549-Galwt cells (Figure 4B), which is consistent with increased cell cycle entry following genistein treatment. It should be noted that expression of INK-family inhibitors ($p15^{INK4B}$, $p16^{INK4A}$ and $p19^{INK4D}$), known to inhibit cyclin D_1 activity, was neither detected nor altered in BT549-Galwt cells (data not shown). While $p21^{WAF1/CIP1}$ is a universal inhibitor of cyclin-dependent kinases and causes cell cycle arrest at G_1/S or at G_2/M, $p27^{KIP1}$ is mostly involved at the G_1/S checkpoint, but not G_2/M (31). Although galectin-3 increased the basal level of $p27^{KIP1}$, $p27^{KIP1}$ expression is drastically down-regulated in BT549-Galwt cells following genistein treatment (Figure 4C), in agreement with rapid cell cycle progression beyond the G_1/S checkpoint. Genistein treatment of BT549-Galwt cells further induced $p21^{WAF1/CIP1}$ expression as shown in Figure 4B. Collectively, these results suggest that galectin-3 induction of $p21^{WAF1/CIP1}$ is in part responsible for genistein-mediated cell cycle arrest at G_2/M in BT549 cells. It should be noted that genistein had no effect on $p21^{WAF1/CIP1}$ expression in BT549neo and BT549-Galm cells, both apoptosis-prone cells. This clearly suggests that $p21^{WAF1/CIP1}$ expression is not required for genistein-induced apoptosis.

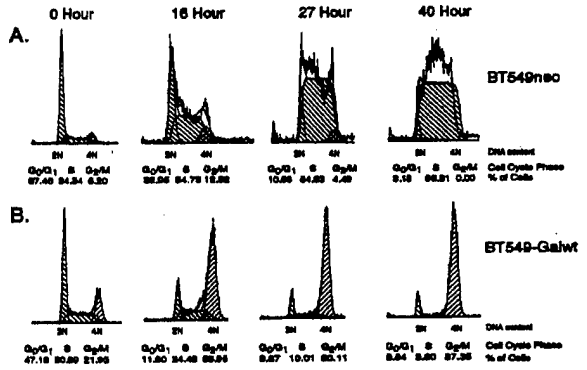

Fig. 3. Galectin-3 mediates cell cycle arrest at G_2/M arrest. Flow cytometric cell cycle histograms of BT549neo (A) and BT549-Galwt (B) treated with 90 μM genistein for 0, 16, 27 and 40 h. The proportions of cells in each cell cycle phase are presented.

Discussion

Increasing evidence suggests that apoptosis regulation is tightly linked to cell cycle regulation. Although apoptosis can be induced at any point during the cell cycle, apoptosis sensitivity varies greatly at different points in the cell cycle. Genistein has previously been shown to induce cell cycle arrest at G_2/M and apoptosis in many cancer cell lines (4,10,16). It has been suggested that $p21^{WAF1/CIP1}$ is critical for both genistein-induced cell cycle arrest and apoptosis. The present study, however, indicates that genistein-induced $p21^{WAF1/CIP1}$ may be associated with apoptosis inhibition through cell cycle arrest rather than apoptosis induction. Our study clearly suggests that the level of expression of anti-apoptotic gene products such as galectin-3 is a critical determinant of genistein-induced cell cycle arrest or apoptosis. This is of particular importance in the light of the recently reported potential carcinogenic activity of genistein (14,15). Genistein-induced apoptosis seems to be accompanied by induction of cell cycle entry (Figure 3). When genistein-induced apoptosis is inhibited by

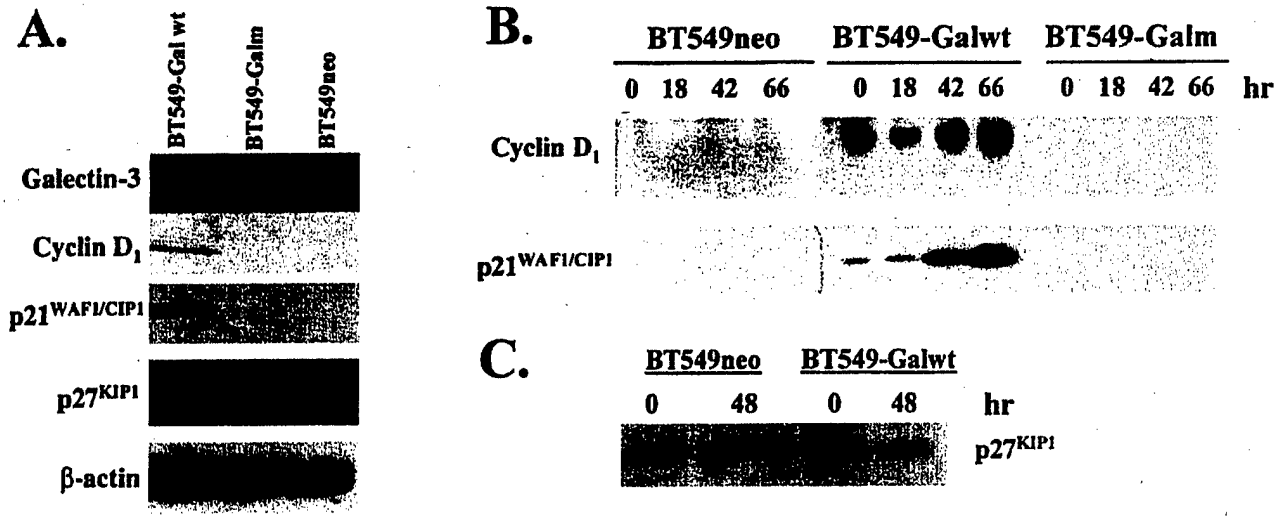

Fig. 4. Galectin-3 induction of $p21^{WAF1/CIP1}$ is associated with G_2/M arrest. Immunoblot analysis of galectin-3, cyclin D_1, $p21^{WAF1/CIP1}$, $p27^{KIP1}$ and β-actin in BT549neo, BT549-Galwt or BT549-Galm cells. Protein samples were prepared from cells without treatment (A) or with 90 μM genistein treatment for the indicated times (B and C).

galectin-3, galectin-3 induction of cyclin D_1 and rapid cell cycle progression beyond the S phase may enhance accumulation of genetic mutations contributing to carcinogenesis.

Previous studies and the present one suggest that galectin-3 results in cell cycle arrest at different points depending on the apoptotic stimuli. Galectin-3 induces cell cycle arrest at late G_1 in response to cisplatin treatment or loss of cell adhesion (anoikis) (27,28), whereas it induces G_2/M arrest following genistein treatment. At present it is not known how galectin-3 modulates the expression of cell cycle regulatory genes, including cyclin D_1, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$. Galectin-3 is expressed in the nucleus and cytoplasm and also in secreted form. Galectin-3 in the nucleus may be involved in the regulation of gene expression. It is equally possible that cytoplasmic galectin-3 or extracellular galectin-3 induces signal transduction leading to modulation of gene expression. Cisplatin treatment or anoikis further enhanced $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ expression in galectin-3-expressing cells, resulting in G_1/S arrest. In contrast, genistein enhanced $p21^{WAF1/CIP1}$ expression only, while it abolished galectin-3 induction of $p27^{KIP1}$, leading to G_2/M arrest. Specific interactions between apoptosis initiation signaling and galectin-3 regulation of gene expression remain to be fully investigated.

In summary, the present study provides a mechanistic insight into genistein-induced cell cycle arrest or apoptosis regulated by the anti-apoptotic gene product galectin-3. Taken in conjunction with the finding that galectin-3 is often overexpressed in human cancer (18–20,22–25), our finding may be critical for understanding the chemopreventive/chemotherapeutic potentials of genistein.

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Appendix 4

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Galectin-3 Protects Human Breast Carcinoma Cells against Nitric Oxide-Induced Apoptosis

Implication of Galectin-3 Function during Metastasis

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Galectin-3 is a β-galactoside-binding protein which regulates many biological processes including cell adhesion, migration, cell growth, tumor progression, metastasis, and apoptosis. Although the exact function of galectin-3 in cancer development is unclear, galectin-3 expression is associated with neoplastic progression and metastatic potential. Since studies have suggested that tumor cell survival in microcirculation determines the metastatic outcome, we examined the effect of galectin-3 overexpression in human breast carcinoma cell survival using the liver ischemia/reperfusion metastasis model. While the majority of control cells died by hepatic ischemia/reoxygenation, nearly all of galectin-3 overexpressing cells survived. We showed that galectin-3 inhibits nitrogen free radical-mediated apoptosis, one of the major death pathways induced during hepatic ischemia/reperfusion. Galectin-3 inhibition of apoptosis involved protection of mitochondrial integrity, inhibition of cytochrome *c* release and caspase activation. Taking these results together with the previous observation that galectin-3 inhibits apoptosis induced by loss of cell adhesion, we propose that galectin-3 is a critical determinant for anchorage-independent and free radical-resistant cell survival during metastasis. ***(Am J Pathol 2001, 159:1055–1060)***

Tumor cell metastasis is a complex process involving motility, invasion, and cell growth and survival. It was observed that the ability of tumor cells to colonize the liver was shown to be proportional to the number of tumor cells surviving within the sinusoid.[1,2] As tumor cells arrest in the hepatic sinusoid and terminal portal venules, microscopic infarcts develop, followed by re-establishment of blood flow. Ischemia/reperfusion results in oxygen and nitrogen radical formation that is toxic to the majority of the arrested tumor cells.[3,4] However, the survival rate of highly metastatic human carcinoma cells is 10-fold higher than that of weakly metastatic cells during hepatic ischemia and reperfusion.[3,4] Presently, the molecular mechanisms by which metastatic tumor cells become resistant to oxygen and nitrogen radicals during hepatic ischemia/reperfusion remain unclear.

Galectin-3 is a 30-kd protein member of the β-galactoside-binding family that is highly expressed in various human tumor cells.[5–9] *In vitro*, galectin-3 modulates a variety of biological processes such as cell adhesion, migration, cyst formation, secretion of cytokines, and pre-mRNA splicing.[10–15] Although the exact function of galectin-3 in cancer development is unclear, its expression is associated with neoplastic progression and metastatic potential[5–9] in head and neck,[10] thyroid,[16] gastric,[6] and colon cancers.[17]

Recently, we and others showed that galectin-3 protects cells against apoptosis.[18–21] Galectin-3 inhibits breast epithelial cell apoptosis induced by a variety of stimuli including staurosporine, cisplatin, and loss of cell adhesion. Similarly, galectin-3 prevents T-cell apoptosis induced by anti-Fas-antibody.[18] Inflammatory cells from galectin-3 deficient-mice were more prone to undergo apoptosis, re-emphasizing a galectin-3 role for apoptosis regulation.[22] In this study, we questioned whether galectin-3 protects tumor cells from oxidative stress-induced apoptosis during metastasis. To test this, we used a mouse ischemic liver tissue/human breast tumor cell co-culture system to examine whether galectin-3 protects human breast carcinoma cells from death during hepatic ischemia-reperfusion. We also investigated the effect of galectin-3 on nitric oxide-induced apoptosis, one of the

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major death pathways induced during hepatic ischemia-reperfusion.

Materials and Methods

Cell Culture

The human breast carcinoma cell line BT549 was obtained from Dr. E. W. Thompson, Vincent T. Lombardi Cancer Research Center, Georgetown University Medical Center, Washington, DC. Galectin-3-transfected BT549 cell clones were previously established by introducing an expression vector containing human galectin 3 cDNA into BT549 parental cells.[19,20] Galectin-3-transfected BT549 and neo-resistant control vector-transfected BT549 cells are referred to as BT549Gal-3 and BT549neo, respectively. Cells on tissue culture dishes (Sarstedt, Newton, NC) were grown in DMEM/F-12 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mmol/L L-glutamine and 0.5 μg/ml fungizone in a 95% air and 5% CO_2 incubator at 37°C.

Cell Labeling with Rd-Dx and Calcein AM

BT549Gal-3 and BT549neo cells were labeled with two fluorescing reagents, rhodamine B-isothiocyanate dextran (Rd-Dx; Sigma Chemical Co., St. Louis, MO), with maximum excitation/emission at 530/590 nm, and calcein AM, with maximum/emission at 485/530 nm. Rd-Dx was loaded into BT549 cells by electroporation (Cell-Porator, BRL, Gaithersburg, MD). Cells (5×10^6), suspended in 20 mg/ml Rd-Dx in phosphate-buffered saline (PBS) solution, received an electrical pulse (capacitance: 330 μF and 300 V; load resistance: high Ω; charge rate: fast). The Rd-Dx-loaded cells were incubated for 16 hours at 37°C in complete tissue culture medium, and then adherent cells were recovered by trypsin. The cells were suspended in PBS and incubated with calcein AM (Molecular Probes, Eugene, OR) at a final concentration of 4 μmol/L for 30 minutes at 37°C.

Liver-BT549 Co-Culture

Athymic nude mice purchased from Harlan Sprague-Dawley Inc. (Frederick, MD) were anesthetized with a general inhalant as previously described.[4] A midline laparotomy incision was made under aseptic conditions to expose the liver. To induce ischemia, the portal vein and hepatic artery were clamped with a microvascular clamp for 3 minutes. The liver was excised and immediately placed in chilled DMEM/F-12 on ice and dissected into 1 to 3 mm fragments. The liver fragments were transferred into a 50-ml centrifuge tube and washed once by centrifugation at 400 × *g* for 5 minutes. The fragments were resuspended in 5 ml of the co-culture medium and loaded into a 55-ml Rotating Wall Vessel (RWV; Rotary Cell Culture System, Synthecon, Inc., Houston, TX). BT549 cells pre-labeled with Rd-Dx and calcein AM were then added to the RWV, and the 55-ml RWV chamber was completely filled with the liver fragment co-culture medium [a 1:1 mixture of HepatoZYME-SFM and DMEM/F-12 with 10% FBS and penicillin-streptomycin] to eliminate bubbles. An air pump was connected to the RWV for reoxygenation after liver ischemia. After co-culture, Rd-Dx and calcein AM-labeled cells were detected on a Nikon upright microscope (Nikon Microphot-FXL, Tokyo, Japan) equipped with epi-fluorescence using digital image capture by an 3CCD color video camera (Model DXC-930P, Sony, Tokyo, Japan) .

Mitochondria Staining

Cells were plated on a coverslip in a six-well plate. After 24 hours of apoptosis induction, the cells were incubated with media containing 250 μmol/L MitoTracker Red (Molecular Probes, Eugene, OR) for 30 minutes at 37°C. Cells were washed with PBS and fixed with 3.7% paraformaldehyde in PBS for 15 minutes at 37°C. The coverslips were mounted onto glass plates using 0.1% phenylenediamine and 90% glycerol in PBS. Fluorescent staining of the mitochondrial membrane was examined with a Nikon Labophot microscope fitted with a digital video camera (Photometrics, Tucson, AZ).

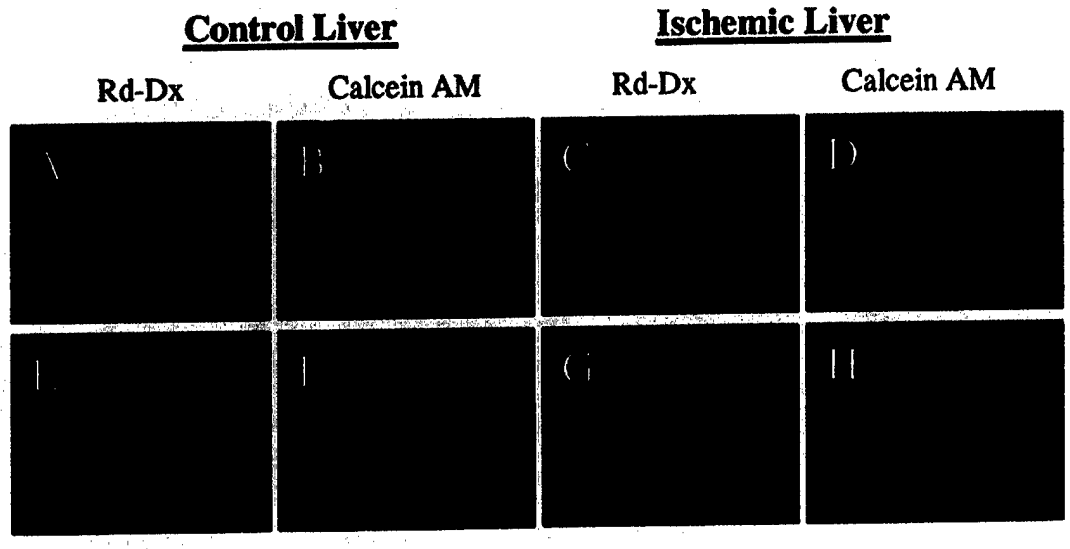
Cytochrome c Release

Cells were harvested at 0, 24, and 48 hours following treatment with 200 μmol/L S-nitroso-*N*-acetylpenicillamine (SNAP; Sigma), resuspended in ice-cold lysis buffer (20 mmol/L Hepes-KOH, pH 7.5, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L sodium EDTA, 1 mmol/L sodium EGTA, 20 μl protease inhibitors (Sigma), 4 mmol/L dithiothreitol, and 1 mmol/L phenylmethylsulfonyl fluoride) containing 250 mmol/L sucrose, and incubated for 1 hour at 4°C. The lysates were passed through a 26 gauge syringe 15 times and then centrifuged at 10,000 rpm for 20 minutes at 4°C. The resulting supernatant was analyzed by immunoblot analysis using anti-cytochrome *c* antibody (ZYMED Laboratories Inc., CA) as previously described.[23]

DEVDase Activity

Cells were collected at 0, 24, 36, 48 and 60 hours after treatment with 200 μmol/L SNAP and lysed in caspase lysis buffer [50 mmol/L Tris buffer (pH 7.5), 0.03% Nonidet and 1 mmol/L DTT]. Nuclei were removed by low speed centrifugation (800 × g, 5 minutes), and the cytosolic fraction was incubated with 40 μmol/L DEVD-amc, 10 mmol/L HEPES (pH 7.5), 50 mmol/L NaCl, and 2.5 mmol/L DTT for 2 hours at 37°C. Fluromethylcoumarin fluorescence, released by DEVDase (caspase) activity, was measured using 380 nm excitation. A CCD device (Instapec IV; Oriel, Straford, CT) fitted with a monochromator was used to measure the fluorescence emission spectrum. The intensity at the optimum (~450 nm) was measured. DEVDase activity was normalized per micro-

(A)



(B)

Cell line	Liver Fragments	Treatment	Viability(%)
BT549 neo	Control		97.2 ± 7.9
BT549 neo	Ischemia		61.6 ± 9.4
BT549 neo	Ischemia	NMMA	92.2 ± 6.1
BT549 Gal-3	Control		96.9 ± 9.8
BT549 Gal-3	Ischemia		100

Figure 1. Galectin-3 protects BT549 cell death induced by free radicals generated during hepatic ischemia/reperfusion. **A:** Rd-Dx and calcein AM-labeled BT549neo (**top panels**) and BT549Gal-3 (**bottom panels**) cells were co-cultured with the liver fragments isolated from control mouse liver (Control) or ischemic liver (Ischemic). Rd-Dx staining (**A, C, E, G**) and calcein AM staining (**B, D, F, H**) were detected with a maximum excitation/emission at 530/590 nm, and at 485/530 nm, respectively. **B:** After co-cultures of BT549neo or BT549Gal-3 cells with liver fragments, the numbers of cells containing Rd-Dx and calcein AM fluorescence were counted. The percentage of cell viability was determined by number of cells containing both Rd-Dx and calcein AM fluorescence/number of cells containing Rd-Dx fluorescence. For NMMA treatment, mice received injections with 20 mg of NMMA via tail vein 30 minutes before liver harvest. Co-cultures were incubated in the presence of 1 mmol/L NMMA. The assay was performed in triplicate and the results are presented as the mean ± SE.

gram of protein determined by BCA protein assay kit (Pierce, Rockford, IL).

Results

To study the role of galectin-3 in inhibiting human breast carcinoma cell death during liver ischemia/reperfusion, we cultured BT549Gal-3 or BT549neo cells in a rotating suspension culture system containing mouse liver fragments, a co-culture system previously shown to maintain the architecture and viability of the liver for at least 24 hours.[4] Hepatic ischemia was induced by clamping the hepatic arterial and portal vein, and an air pump was connected to the rotating suspension culture system to mimic the *in vivo* reoxygenation after liver ischemia. To distinguish human breast carcinoma cells from mouse liver cells, the human cells were labeled with Rd-Dx. The previous study[24] demonstrated that Rd-Dx is nontoxic, does not leach out of cell cytoplasm, and retains its fluorescence until diluted by cell division. To distinguish viable cells after the co-culture period, the human cells were also prelabeled with calcein AM, since it produces fluorescence signal only in live cells with ATP-dependent cytoplasmic esterase activity. As shown in Figure 1, control mouse liver cells were not toxic to human breast carcinoma cells. When BT549neo cells were co-cultured with ischemic liver fragments, only 62% of the cells survived at 24 hours. In contrast, all of BT549Gal-3 cells survived after 24 hours of co-culture with ischemic liver fragments. These results imply that galectin-3 expression in human breast carcinoma cells protects against cell death during liver ischemia/reperfusion.

Previous studies showed that reactive nitrogen free radicals generated during liver ischemia/reperfusion are the major toxic molecules for the weakly metastatic colorectal cancer cells.[4] Here, we examined whether the

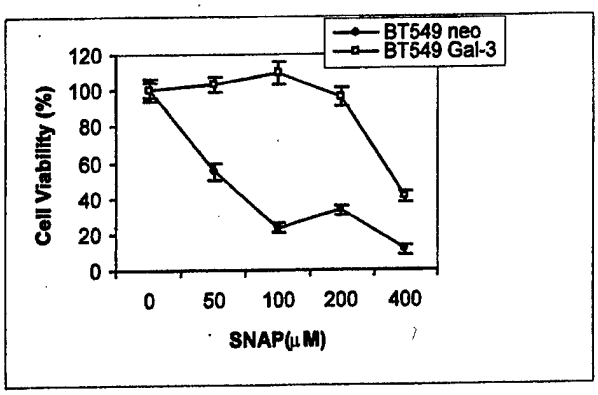


Figure 2. Galectin-3 inhibits SNAP-induced cell death in human breast epithelial cells. BT549neo and BT549Gal-3 cell cultures in the presence of 0, 50, 100, 200, or 400 μmol/L SNAP for 48 hours. The number of live cells was determined by trypan blue exclusion assay. The percentage of cell viability was normalized to the respective untreated cells. All experiments were performed in triplicate and the error bars represent the SD.

same molecular species are responsible for killing BT540neo cells. To this end, mice were pretreated by a tail vein injection with 20 mg of N^G-monomethyl-L-arginine (NMMA), a NO synthase inhibitor, for 30 minutes before ischemic treatment. The isolated liver fragments were co-cultured with BT549neo cells in the presence of 1 mmol/L NMMA. The cell viability with NMMA treated ischemic liver fragments increased to the comparable levels of co-culture with control mouse liver fragments. These suggested that reactive nitrogen free radicals generated during hepatic liver ischemia/reperfusion were cytotoxic to human breast carcinoma BT549 cells similarly to colorectal cancer cells, and that galectin-3 protects BT549 cells against nitrogen free radical-induced cell death.

To further study galectin-3 inhibition of cell death induced by reactive nitrogen free radicals, we examined the role of galectin-3 in SNAP-induced cell death. SNAP is a NO donor known to induce cell death in many cell types including skeletal myoblasts, cortical neuronal cells, hepatocytes, and smooth muscle cells [25–27]. Substantial cytotoxicity was induced by SNAP in BT549neo cells (Figure 2). After 48 hours of treatment with 100 μmol/L SNAP, approximately 80% of BT549neo cells died, whereas no significant cytotoxicity was detected in BT549Gal-3 cells following up to 200 μmol/L SNAP treatment for 48 hours. This demonstrated that galectin-3 expression protects human breast epithelial cells from nitric oxide-induced cell death. Next we investigated whether galectin-3 regulates the nitric oxide-induced apoptotic pathway. It is now well established that mitochondria and caspases play central roles in apoptosis regulation [28,29]. Mitochondrial events critical for apoptosis include disruption of electron transport, loss of mitochondrial transmembrane potential (Ψ_m), and release of cytochrome *c*.[28,29] To examine whether galectin-3 protects mitochondria integrity, we stained BT549neo and BT549Gal-3 cells with MitoTracker Red which selectively stains mitochondria and serves as a marker for the mitochondrial membrane potential.[23] Twenty four hours of treatment with 200 μmol/L SNAP resulted in loss of mitochondria structure in ~90% of BT549neo cells. In contrast, more than 95% of the mitochondria in SNAP-treated BT549Gal-3 cells retained the fibrillar fluorescence pattern observed in the untreated cells, suggesting that galectin-3 overexpression protects cells against the loss of $\Delta\Psi_m$ (Figure 3A). As predicted from the loss of mitochondrial integrity (Figure 3A), immunoblot analysis of cytosolic cytochrome *c* showed that the level of cytochrome release from the mitochondria was elevated in BT549neo cells as compared with BT549Gal-3 cells following 200 μmol/L SNAP treatment (Figure 3B).

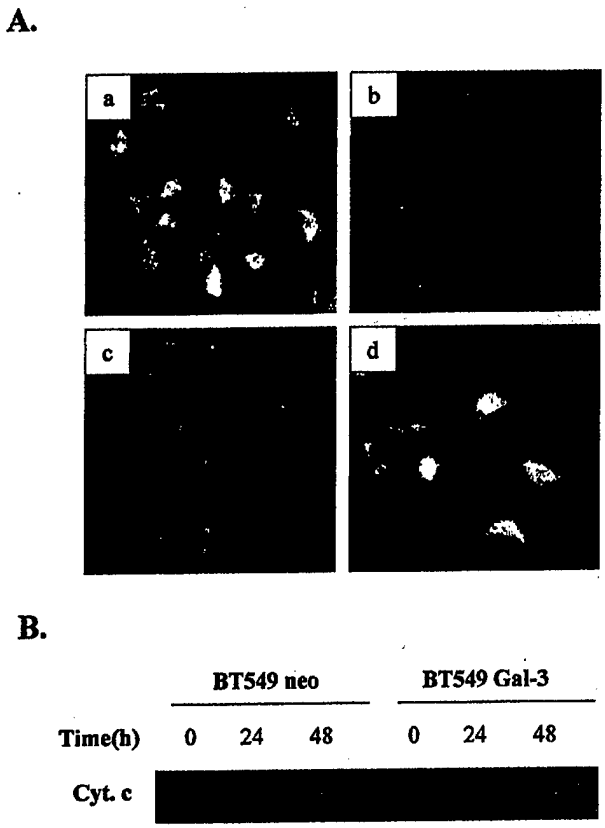


Figure 3. Galectin-3 protects mitochondria integrity and prevents cytochrome *c* release. **A:** BT549neo (**a, b**) and BT549Gal-3 (**c, d**) cells were plated on coverslips in six-well plates. Cells were treated with 0 (**a, c**) or 200 μmol/L SNAP (**b, d**) for 24 hours and stained with a fluorescent probe, MitoTracker Red. The mitochondria staining was examined with a Nikon Labophot microscope fitted with a digital video camera. **B:** Immunoblot analysis of cytosolic cytochrome *c* in BT549neo and BT549Gal-3 cells following 200 μmol/L SNAP treatment for 0, 24, or 48 hours.

Cytosolic cytochrome *c* translocated from the mitochondria was shown to be critical for caspase activation, a group of cysteine proteases that initiate the apoptotic process.[30,31] To further examine the effect of galectin-3 on nitric oxide-induced apoptosis, we measured caspase activity using the flurogenic substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (Ac-DEVD-amc), a substrate for caspase-3, -6, -7, -8, and -10. Caspase (DEVDase) activity was determined by amc release from the tetrapeptide substrate Ac-DEVD-amc. While SNAP treatment induced DEVDase activity ~fourfold in BT549neo cells following SNAP treatment, there was no induction in BT549Gal-3 cells following

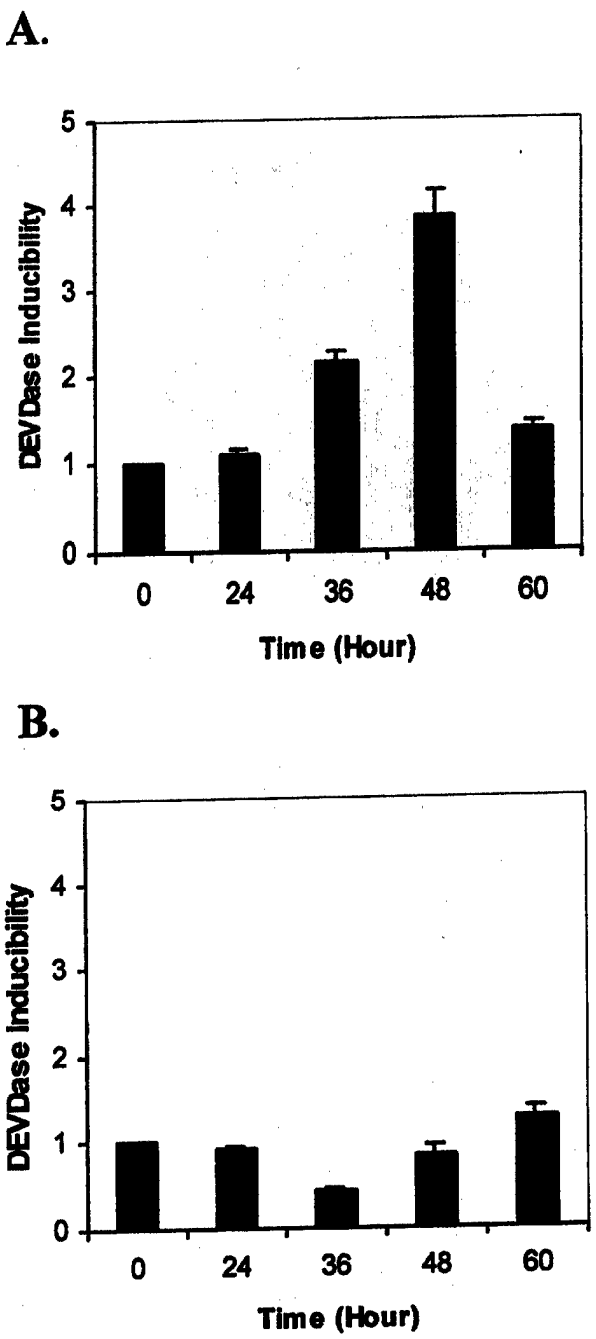


Figure 4. Galectin-3 inhibits DEVDase activity. BT549neo (**A**) and BT549Gal-3 (**B**) cells grown in 60-mm dishes were treated with 200 μmol/L SNAP. At indicated time points, the cells were washed with PBS and lysed with 200 μl caspase buffer as described in Materials and Methods. DEVDase activity in cytosol was determined by amc release from the tetrapeptide substrate Ac DEVD-amc, and DEVDase activity was normalized per μg protein. The DEVDase inducibility was normalized to the respective untreated cells. Three independent experiments were performed and the error bars represent SD of the mean of triplicates.

the same treatment (Figure 4). These results demonstrate that galectin-3 inhibition of apoptotic cell death involves protection of mitochondria integrity, prevention of cytochrome *c* release, and inhibition of caspase activation.

Discussion

Although recent advances in combined treatment modality including surgery, radio- and chemotherapy significantly improved the local control of breast cancer progression, most deaths from the disease still result from metastasis to the bone, lung, brain, and liver.[32] In liver metastasis, tumor cells enter the hepatic microcirculation mainly by size restriction, and are arrested in the hepatic sinusoids, where they may obstruct blood flow.[33–36] This appears to cause microscopic infarcts within the liver parenchyma located primarily near the capsule of the liver. *In vivo* and *in vitro* studies strongly suggest that the majority of tumor cells die in the microcirculation during reoxygenation of ischemic liver, mostly by reactive nitrogen free radicals.[4] The present study showed that galectin-3 may rescue human breast epithelial cells from nitrogen free radical-induced cell death during liver ischemia/reperfusion. Previously, we also demonstrated that galectin-3 inhibits apoptosis induced by loss of cell adhesion.[20] Taken together, we propose that galectin-3 is a critical determinant for anchorage-independent and free radical-resistant cell survival of disseminating cancer cells in the circulation during metastasis. This might explain why enhanced galectin-3 expression is often associated with the metastatic phenotype.[5–10,16,17]

Galectin-3 inhibits the intrinsic apoptotic pathway in breast epithelial cells induced by a variety of stimuli including nitric oxide, staurosporine, cisplatin, and loss of cell adhesion.[19–21] The ability of galectin-3 to protect cells against apoptosis induced by agents working through different mechanisms suggests that galectin-3 probably acts in a common central pathway of the apoptotic cascade, involving protection of mitochondrial integrity and caspase inhibition. Consistent with our study, Matarrese et al recently suggested that galectin-3 may be a mitochondrial-associated apoptotic regulator.[37] Thus, galectin-3 appears to be a potent inhibitor of mitochondria-mediated intrinsic apoptosis in breast epithelial cells. Interestingly, however, galectin-3 fails to prevent breast epithelial cell apoptosis induced by TRAIL (unpublished data), suggesting that galectin-3 may not be a general inhibitor of extrinsic apoptosis. Structurally, galectin-3 is composed of three distinct domains: an N-terminal leader domain of 12 amino acids containing serine/threonine phosphorylation sites, proline and glycine rich domain, and a globular C-terminal domain containing the carbohydrate recognition site.[5,38] Galectin-3 contains 4 amino acid residues (NWGR) that are conserved in the BH1 domain of the anti-apoptotic members of the bcl-2 gene family. This motif was shown to be critical for bcl-2 anti-apoptotic activity.[39] Similarly, substitution of the Gly residue with Ala in the NWGR motif of galectin-3 abrogates its anti-apoptotic function.[19,20] Galectin-3 may interact with apoptosis-regulating gene products through the NWGR motif and thus replace or mimic bcl-2 activity. Alternatively, galectin-3 may interact with bcl-2 family members and modulate their activity. The molecular mechanisms by which galectin-3 regulates intrinsic and extrinsic apoptosis are now under investigation.

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