Retinoblastoma Function Is a Better Indicator of Cellular Phenotype in Cultured Breast Adenocarcinoma Cells than Retinoblastoma Expression

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Loss of or lowered retinoblastoma (Rb) expression has been included as a prognostic indicator in breast cancer. Low or no Rb expression is seen most commonly in high-grade breast adenocarcinomas, suggesting that a relationship may exist between loss of Rb and a less differentiated state, high proliferation rate, and high metastatic potential. In this study, we compared Rb function in two established breast adenocarcinoma cell lines, MCF-7 and MDA-MB-231, and in an established immortalized mammary epithelial cell line, MCF10A. Cells were synchronized in G0/G1 and were released for several durations, at which time total Rb protein, mRNA, and Rb/E2F/DNA complex formation were evaluated. Rb protein was significantly higher in the tumor cells than in MCF10A cells. However, Rb function was high for a longer duration in MCF10A cells as compared with MCF-7 and MDA-MB-231 cells. Our data support the general conclusion that Rb function, but not necessarily Rb protein, is lower in highly malignant breast adenocarcinoma cells as compared with lower grade tumor cells. These results emphasize the relevance of assessing Rb function over Rb protein. This is particularly important if Rb is to be used as a prognostic indicator for breast adenocarcinoma. [Exp Biol Med Vol. 227(5):354–362, 2002]

Key words: breast adenocarcinoma; cell cycle; gene expression; gene regulation; retinoblastoma

The protein product of the retinoblastoma gene, Rb, is a 110,000-Dalton tumor suppressor protein (1). Rb is often mutated or expressed at very low levels in several tumor types, including retinoblastoma, osteosarcoma, small cell lung carcinoma, and colon, prostate, bladder, and breast carcinomas (2–6). Loss of or lowered Rb expression is more often found in high-grade than in low-grade breast adenocarcinoma tumors and cell lines. A correlation may exist between Rb amounts and differentiated state, metastatic potential, and rate of proliferation of cells (7). As a consequence, Rb has been included as a prognostic indicator in breast cancer (8, 9).

Rb regulates cell cycle progression through mechanisms that transcriptionally downregulate the activity of genes required for progression into S phase (10, 11). Rb binding specifically inhibits E2F activity by blocking the transcriptional activation domain and/or recruiting histone deacetylase activity (12–16). Inactivation of Rb by mutations in the structural gene, transcriptional inactivation through hypermethylation of the promoter, post-transcriptional mechanisms, and post-translational inactivation through hyperphosphorylation have been found to occur in various tumor types (5, 17, 18).

The majority of previous investigations of Rb in breast adenocarcinoma tumors and cell lines have used methods, including immunohistochemistry and immunoblotting, to determine relative amounts of Rb. Functional assays have not been used. In this study, cell cycle-dependent Rb protein expression and Rb function were compared in three cell lines: MCF10A, spontaneously immortalized mammary epithelial cells (19); MCF-7, moderately differentiated, estrogen receptor-positive, non-metastatic mammary-infiltrating ductal adenocarcinoma cells (20); and MDA-MB-231, poorly differentiated, estrogen receptor-negative, highly metastatic mammary adenocarcinoma cells (21). In addition to representing multiple phenotypes of breast adenocarcinoma, these cell lines also express different levels of G1 cyclins and cdk inhibitors, and are therefore hypothesized to

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have varying degrees of Rb function. MCF10A cells express normal levels of cyclins and cdk inhibitors; MCF-7 cells express high levels of cyclin E; and MDA-MB-231 cells express high levels of three G1 cyclins (D1, D3, and E) and low levels of the cdk inhibitor p21 (21–24). Based on this information, we hypothesized that MCF10A cells have somewhat normal Rb function, whereas the tumor cell lines have reduced Rb function in comparison. We also hypothesized that MDA-MB-231 cells will have the most reduced Rb function of the three cell lines.

Materials and Methods

Cell Culture. All chemicals and reagents, tissue culture chemicals, and media were obtained from Sigma Chemical Co. (St. Louis, MO) except for fetal bovine serum (FBS), which was obtained from Atlanta Biologicals (Atlanta, GA), and equine serum, which was obtained from Gibco-BRL (Gaithersburg, MD). Disposables for tissue culture (Falcon and Nunc) were obtained through VWR (Houston, TX). MCF-7 and MDA-MB-231 cells (American Type Culture Collection [ATCC], Manassas, VA) were grown in modified Eagle's medium (MEM) supplemented with 10% FBS. Hs578Bst mammary myoepithelial cells (ATCC) were grown in Dulbecco's MEM (DMEM) /F12 media supplemented with 30 ng/ml epidermal growth factor (EGF) and 10% FBS. MCF10A cells (Karmanos Cancer Foundation, Detroit, MI) were grown in DMEM /F12 supplemented with 5% equine serum, 20 ng/ml epidermal growth factor (EGF), 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, 100 μg/ml streptomycin, and 100 units/ml penicillin. Cells were incubated at 37°C with 5% CO_2.

Isolation of Genomic DNA and Southern Analysis. Genomic DNA (10 μg) isolated using the QIAmp kit (Qiagen, Valencia, CA) was digested with 5× excess restriction enzyme HindIII or EcoRI (Promega, Madison, WI) overnight at 37°C. Digests were electrophoresed on an agarose gel and were Southern blotted using standard methods (25). Hybridization was performed with 1 to 5×10^6 cpm/ml of random primer-labeled probe [3.8R or GA3PDH cDNA probe; ATCC, Random Primer Labeling kit; Boehringer-Mannheim, Indianapolis, IN; α-^{32}P-dCTP (3000 mCi/m*M*); DuPont-NEN Research Products, Boston, MA; and Hybond-N and Rapid Hyb; Amersham, Arlington Heights, IN] at 65°C for 2 hr. Membranes were washed at room temperature with 2× SSC/0.1% SDS twice for 20 min and were exposed to film at −80°C for 2 days with an intensifying screen. Blots were performed for each restriction enzyme at least twice with independent DNA samples.

PCR and Analysis of PCR Products. Oligonucleotide primers, RbproA (5′-CAGCGCTCCAAGTTTG-3′) and Rb-4 (5′-TCCCGACTCCCGTTACAA-3′) (26) were synthesized by Gene Technologies Laboratory at Texas A&M University. PCR was performed according to conditions previously described (26). Twenty microliters of each PCR reaction was electrophoresed, and product sizes were compared. A 100-bp ladder was used as the molecular weight standard. Isolated PCR products were submitted to the Gene Technologies Laboratory at Texas A&M University for automated sequencing with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA). At least two independent samples from each cell line were sequenced both forward and backward.

Cell Synchronization by Serum Depletion. Cells were plated at a density of 1.6×10^4 cells/cm^2 for tumor cells and 2.8×10^4 cells/cm^2 for MCF10A cells. Tumor cells were plated in low serum (2.5%) containing media for 2 to 3 days. The cells were rinsed twice with DPBS and were incubated in serum-free media with 0.5 mg/ml bovine serum albumin (BSA) for 72 hr. MCF-7 cells were incubated in phenol red-free media during serum deprivation. MCF10A cells were serum and growth factor deprived for 48 hr. At the end of serum deprivation, cells were rinsed twice with sterile DPBS, and the appropriate media containing serum and/or growth factors was added in all samples except for those used for the 0-hr time point. At the respective time points, cells were trypsinized and rinsed once with DPBS, collected by centrifugation, and stored at −80°C as cell pellets.

Flow Cytometric Analysis of Cell Cycle Phase Distribution. Cells were harvested at the appropriate times and were fixed by dropwise addition of 1 ml of cold (−80°C) methanol to 1 million cells in 100 μl of residual DPBS while vortexing gently. Cells were stored in methanol at −20°C for at least 30 min prior to staining. Cells were then washed in DPBS at 4°C and were incubated with RNase (360 U per million cells; Worthington Biochemical, Freehold, NJ) for 20 min at 37°C. Cells were pelleted by centrifugation, were resuspended in 1 ml of cold DPBS with 50 μg/ml propidium iodide, and were incubated in amber tubes for 2 hr at 4°C. Cells were filtered through spectramesh filter (Fisher Scientific, Pittsburgh, PA) or passed through a 25-gauge needle. DNA content was measured by a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and was analyzed with MODFIT LT software (Verity Software House, Topsham, ME). Flow cytometric experiments were performed at least three independent times for each cell line.

Solubilization of Cell Pellets for Protein and Immunoblotting. Antibodies recognizing Rb (IF8), E2F-1 (sc-20X), and E2F-4 (sc-20X) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For Rb immunoblots, cell pellets were solubilized as previously described (27). Supernatants were assayed for protein concentration by the modified Lowry protocol (28). Protein (150 μg) was separated by SDS-PAGE (7.5%) and was immunoblotted using primary anti-Rb (IF8) antibody (1:100 dilution) or anti-actin (ICN Biochemicals, Aurora, OH) monoclonal antibody (1:400 dilution) followed by incubation with alkaline phosphatase-conjugated goat anti-mouse secondary antibody (1:3000; Bio-Rad, Hercules, CA). Blots were devel-

oped using the Bio-Rad Alkaline Phosphatase kit as previously reported (27).

For E2F-1 and E2F-4 immunoblots, 50 μg of nuclear protein (prepared as described below) from each cell line was electrophoresed on a 10% denaturing polyacrylamide gel. Proteins were transferred and blotted as previously described with anti-E2F-1 or anti-E2F-4 antibodies (1:5000 dilution) and were developed as above.

Northern Analysis. Total RNA was extracted from harvested cells by the guanidinium thiocyanate method (29). RNA was spectrophotometrically quantified, separated on a 1.2% denaturing formaldehyde agarose gel, and transferred to a Genescreen nylon membrane by capillary diffusion. Random primer-labeled 0.9-kbp RB probe (30) ($1–5 \times 10^6$ cpm/ml) was hybridized in RapidHyb at 65°C for 2 hr. Membranes were washed at room temperature with 1× (0.15 M sodium chloride, 0.01 M sodium phosphate, 0.001 M EDTA, PH 7.4) (SSPE) twice for 15 min each time and were exposed to film at −80°C for 2 days with an intensifying screen.

Analysis of Immunoblots, Northerns, and Southerns. Blots were scanned with a Visage 1400 high-resolution digital camera-based densitometer and were analyzed using BioImage whole band software (Genomic Solutions, Ann Arbor, MI). Scans were performed such that only signals in the linear range of detection were used for analysis. Integrated intensity of Rb signal was normalized to the integrated intensity of corresponding actin signal (for immunoblots) or glyceraldehyde 3-phosphate dehydrogenase (GA3PDH) signal (for Northerns and Southerns) to determine Rb protein, mRNA, and DNA levels, respectively. Analysis of immunoblots and Northerns included determining the ratio of the intensity of the normalized Rb signal from MCF-7 and MDA-MB-231 cells for each time point to the respective normalized Rb signal of MCF10A cells. To normalize for loading and transfer variances, the sum of the density of RB bands on the Southern blot (3.8 R probe) for each sample was divided by the sum of the density of the respective GA3PDH bands. As with immunoblots and Northerns, a ratio of the normalized Rb signal was made for each of the tumor lines to MCF10A cells. Ratioed values for three independent experiments were statistically analyzed using analysis of variance (ANOVA) at $P < 0.05$ and were reported in graphical format or discussed in "Results."

Nuclear Extract Preparation and Gel Mobility Shift Assays. γ-^{32}P-ATP (3000 mCi/m*M*) was purchased from Dupont-NEN. E2F-1 consensus (5′-ATTTAAGT*TTCGCGC*CCTTTCTCAA-3′) and mutant (5′-ATTTAAGT*TTCGATC*CCTTTCTCAA-3′) oligonucleotides were purchased from Santa Cruz Biotechnology. Chroma spin TE-10 columns were purchased from Clontech (Palo Alto, CA). Nuclear extracts were prepared in a high-salt (0.5 *M* KCl) buffer using standard methods (31). Nuclear extracts were dialyzed against 20 m*M* HEPES, 0.1 m*M* EGTA, 10% glycerol, 1.0 m*M* $MgCl_2$, 50 m*M* KCl, 1 m*M* DTT, and 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF) for 1 to 1.5 hr at 4°C. Extracts were assessed for protein by the Bradford assay and were stored at −80°C. Nuclear extract (8 μg) was incubated with 1 μg/ml denatured herring sperm DNA, 500 μg/ml BSA, 20 mM HEPES, 0.1 m*M* EGTA, 10% glycerol, 1.0 m*M* $MgCl_2$, 50 m*M* KCl, 1 m*M* dithiothreitol (DTT), and 0.1 m*M* PMSF for 10 min at room temperature. Fifty molar excess of wild-type or mutant unlabeled E2F oligonucleotide was incubated (for 10 min at room temperature) with extracts as competitive controls for specific and nonspecific binding, respectively. T4 polynucleotide kinase (Promega) end-labeled E2F-1 oligo (60,000 cpm, specific activity 8×10^6 cpm/ng) was added to all reactions and was incubated for 15 min at room temperature. To assay for Rb and/or E2F protein in the protein/DNA complexes, antibodies to E2F-1 (sc-20X), E2F-4 (sc-20X), or Rb (IF8X; 0.5 μg of each, respectively) were added and incubated for 15 min at room temperature. Loading buffer (0.25% xylene cyanol, 0.25% bromphenol blue, and 30% glycerol) was added and samples were electrophoresed through a 4% nondenaturing polyacrylamide gel at 120 V for 3 hr. The gel was dried and exposed to film overnight.

Analysis of Gel Mobility Shift Assays. Autoradiographs from gel mobility shift assays were scanned with a Visage 1400 high-resolution digital camera-based densitometer (Genomic Solutions, Ann Arbor, MI) and were analyzed using BioImage whole band software (Genomic Solutions). Scans were performed such that only signals in the linear range of detection were used for analysis. Ratios of the integrated intensity of the Rb/E2F/DNA complex to the E2F/DNA complexes were averaged for each cell line and were plotted versus time. Statistical analysis was performed with data from three independent experiments using ANOVA at $P < 0.05$ to determine significance from Rb activity at the 0-hr time point.

Results

RB Structural Gene and RB Promoter Are Intact in MCF10A, MCF-7, and MDA-MB-231 Cells. Consistent with a previous report (32), Southern analysis of the RB gene in MCF10A, MCF-7, and MDA-MB-231 cells revealed no large structural mutations. Normalized intensity of RB signal in each of the tumor lines divided by the MCF10A normalized RB signal revealed that the copy number was unchanged between the three lines. With the MCF10A ratio defined as one, ratios observed for MCF-7 (0.894 ± 0.211) and MDA-MB-231 (1.40 ± 0.491) were similar.

Mutations in the RB promoter have been found in prostate tumors and recently in retinoblastomas (26, 33). To assess integrity of the RB promoter, a 472-bp region sufficient for 100% activity (34) (Fig. 1) was PCR amplified from Hs578Bst (normal mammary myoepithelial cells), MCF-7, and MDA-MB-231. Size comparison of PCR products revealed no major deletions (data not shown). No mutations or deletions were found when sequences of the PCR

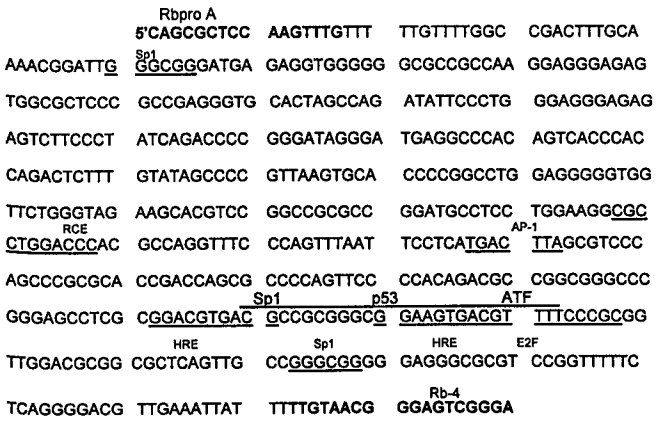

Figure 1. Sequence of the RB promoter region −614 to −142 bp. Primers are in bold, and transcription factor binding sites are underscored or overscored.

products were compared with the reported sequences for this region (35) (data not shown).

Synchronization of MCF10A, MCF-7, and MDA-MB-231 Cells by Serum Deprivation. Because Rb is cell cycle regulated, assessment of Rb protein and mRNA expression required that cells be arrested in G0/G1 and allowed to progress through the cell cycle in a synchronized manner. Cells were stained with propidium iodide (PI) to monitor DNA content. After serum deprivation, approximately 95% of MCF10A cells and approximately 80% of MCF-7 and MDA-MB-231 cells were in G0/G1. Upon addition of serum, cells progressed through the cell cycle in a synchronized manner (Table I). Percentages of cells in S phase were higher overall in the tumor cells than in MCF10A cells. The S-phase peak occurred at 24 to 36 hr after serum addition, followed by a G2/M peak at 36 hr for all three cell lines. This allowed observation of Rb expression in a cell cycle-dependent manner and provided a 12- to 24-hr window to study G1 activities in all three cell lines.

Cell Cycle-Regulated Expression of Rb Protein and mRNA. Using immunoblot analysis of Rb at the times indicated after serum addition, we found no significant cell cycle-dependent changes in Rb protein expression in any of the cell types. Multiple molecular weight species of Rb (Fig. 2A) were observed and may have represented variations in phosphorylation or protein degradation. Because the antibody used for detection was not specific to the C-terminal region of Rb, we could not distinguish between these possibilities (36). There were higher amounts of total Rb protein in the tumor lines as compared with MCF10A cells. This difference was statistically significant (Fig. 2B). We found no significant differences in Rb mRNA expression across the time course or between the three cell lines (Fig. 3). It was then our aim to determine if the statistically significant difference in Rb protein amount was biologically relevant.

Rb Function Varies with Cell Cycle Progression in Each of the Cell Lines. Rb's tumor suppressor activity stems from its ability to bind E2F proteins and actively inhibit transcriptional activation through E2F sites (10, 11). Therefore, cells with high levels of Rb protein, assuming that it is hypophosphorylated, would be expected to have well-controlled cell cycles with limited numbers of

Table I. Flow Cytometric Analysis of Cellular DNA Subsequent to Release of Cell Cycle Arrest

Cell line Time (hr)	%G0/G1	%S	%G2/M
MCF10A			
0	95 ± 0.6	1 ± 0.1	4 ± 0.6
6	95 ± 0.9	1 ± 0.3	4 ± 1.1
12	94 ± 1.1	1 ± 0.3	5 ± 0.9
24	68 ± 13.5	27 ± 12.5	5 ± 1.2
36	51 ± 4.0	17 ± 4.4	32 ± 1.8
48	60 ± 5.9	19 ± 3.7	21 ± 2.7
MCF-7			
0	80 ± 0.6	13 ± 0.4	7 ± 0.5
6	80 ± 1.8	12 ± 1.4	8 ± 0.7
12	79 ± 2.1	11 ± 1.4	10 ± 0.8
24	40 ± 2.5	50 ± 4.4	10 ± 5.0
36	63 ± 6.4	17 ± 3.2	20 ± 3.5
48	69 ± 4.7	21 ± 3.5	10 ± 1.2
MDA-MB-231			
0	81 ± 0.6	10 ± 0.6	9 ± 2.2
6	81 ± 4.9	9 ± 3.3	11 ± 4.4
12	82 ± 4.4	7 ± 4.7	11 ± 4.9
24	53 ± 12.2	36 ± 10.3	11 ± 3.7
36	60 ± 1.0	21 ± 1.3	19 ± 2.0
48	62 ± 5.0	25 ± 2.0	14 ± 3.5

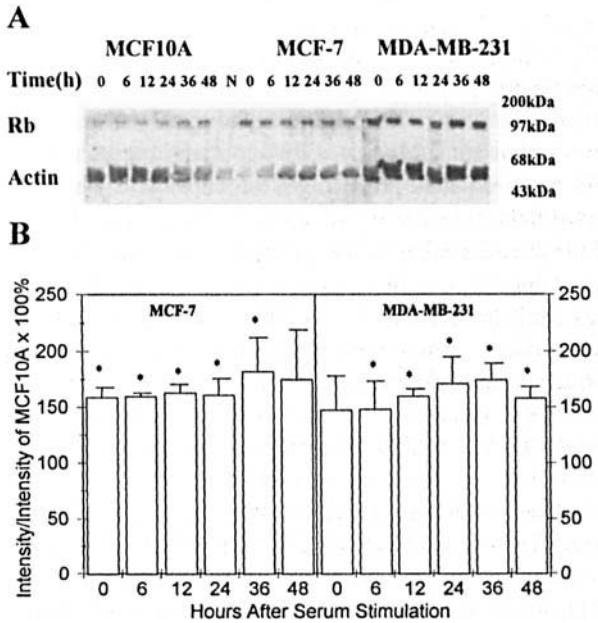

Figure 2. Rb protein expression in MCF10A, MCF-7, and MDA-231 cells during cell cycle progression. Time (hr) indicates period of serum stimulation subsequent to serum deprivation. (A) Representative immunoblot of Rb (105–115 kDa) and actin (49 kDa). Molecular weight standards are indicated. Protein from Rb-deficient MDA-MB-468 cells (N) was used as a negative control. (B) Densitometric analysis of three independent experiments as described in "Materials and Methods." Values (mean ± SD) significantly different from MCF10A values (100%) are indicated by an asterisk.

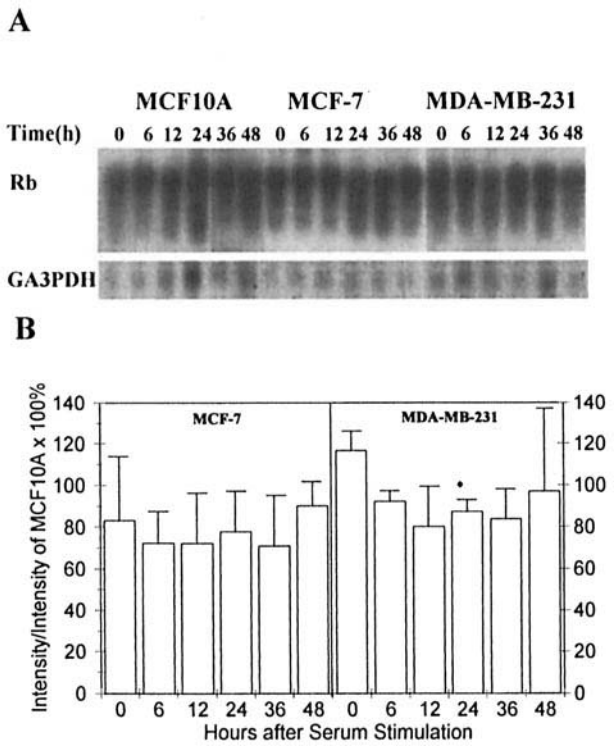

Figure 3. RB mRNA expression in MCF10A, MCF-7, and MDA-231 cells during cell cycle progression. Time (hr) indicates period of serum stimulation subsequent to serum deprivation. (A) Representative Northern blot of RB (4.7 kb) and GA3PDH (2.0 kb). (B) Densitometric analysis of three independent experiments as described in "Materials and Methods." Values (mean ± SD) significantly different from MCF10A values (100%) are indicated by an asterisk.

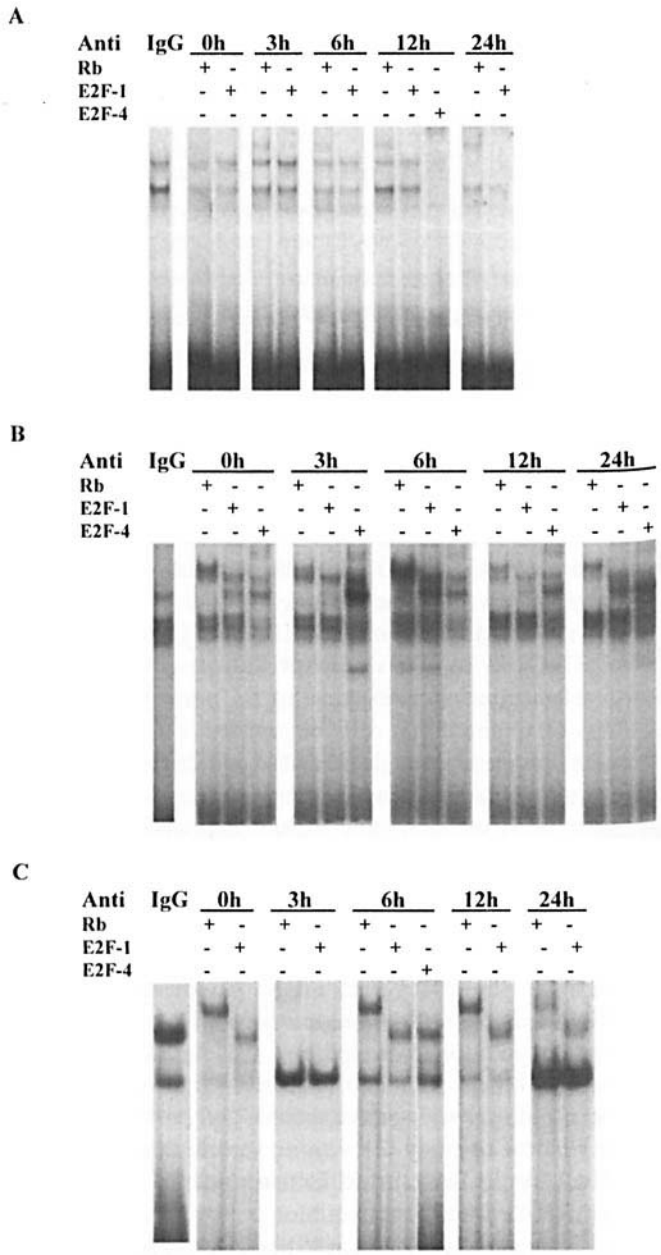

Figure 4. Autoradiographs of gel mobility shift assays using nuclear extracts from MCF10A, MCF-7, and MDA-MB-231 cells and E2F promoter oligonucleotide. Time (hr) after serum stimulation subsequent to serum deprivation is indicated. Antibodies added and IgG control are indicated. (A) MCF10A; (B) MCF-7; (C) MDA-MB-231.

cells in S phase. This is consistent with previous studies showing lowered or loss of Rb expression in high-grade breast adenocarcinoma cells. Despite these predictions, our flow cytometric analysis of DNA content in serum-deprived cells revealed that percentages of cells in S phase were consistently higher in those cells with higher amounts of Rb (MCF-7 and MDA-MB-231 cells). To determine if the higher number of cells in S phase in the tumor lines correlated with lowered or loss of Rb function, we examined Rb/E2F/DNA complex formation in the three cell lines. Gel mobility shift assays were used to monitor functional Rb in nuclear extracts of synchronized cell populations. To identify Rb in the E2F/DNA complex, an Rb antibody was added to the reaction to supershift the Rb/E2F/DNA complex. One band consistently supershifted upon addition of Rb antibodies; addition of a nonspecific (IgG) antibody caused no detectable changes (Fig. 4). Hence, the supershifted band was identified as the Rb/E2F/DNA complex. The banding pattern in our assay is similar to results from previous researchers who have used gel mobility shift assays to identify Rb/E2F/DNA and related complexes in stimulated mononucleocytes and human tumor cell lines (37, 38).

Expression and activity of the five E2F proteins that are transcriptional activators (39), and the Rb family proteins (Rb, p107, and p130) involved in inhibition of E2F activity (11) are all cell cycle dependent. To account for cell cycle-regulated variations in total E2F activity within and across cell lines, as well as possible loading differences, the intensity of the Rb/E2F/DNA complex (highest molecular weight band in MCF10A and MDA-MB-231 cells, and second band from the top in MCF-7 cells) was divided by the total intensity of the E2F/DNA complexes (the single lower molecular weight bands for MCF10A and MDA-MB-231 cells and the three lowest molecular weight bands for MCF-7

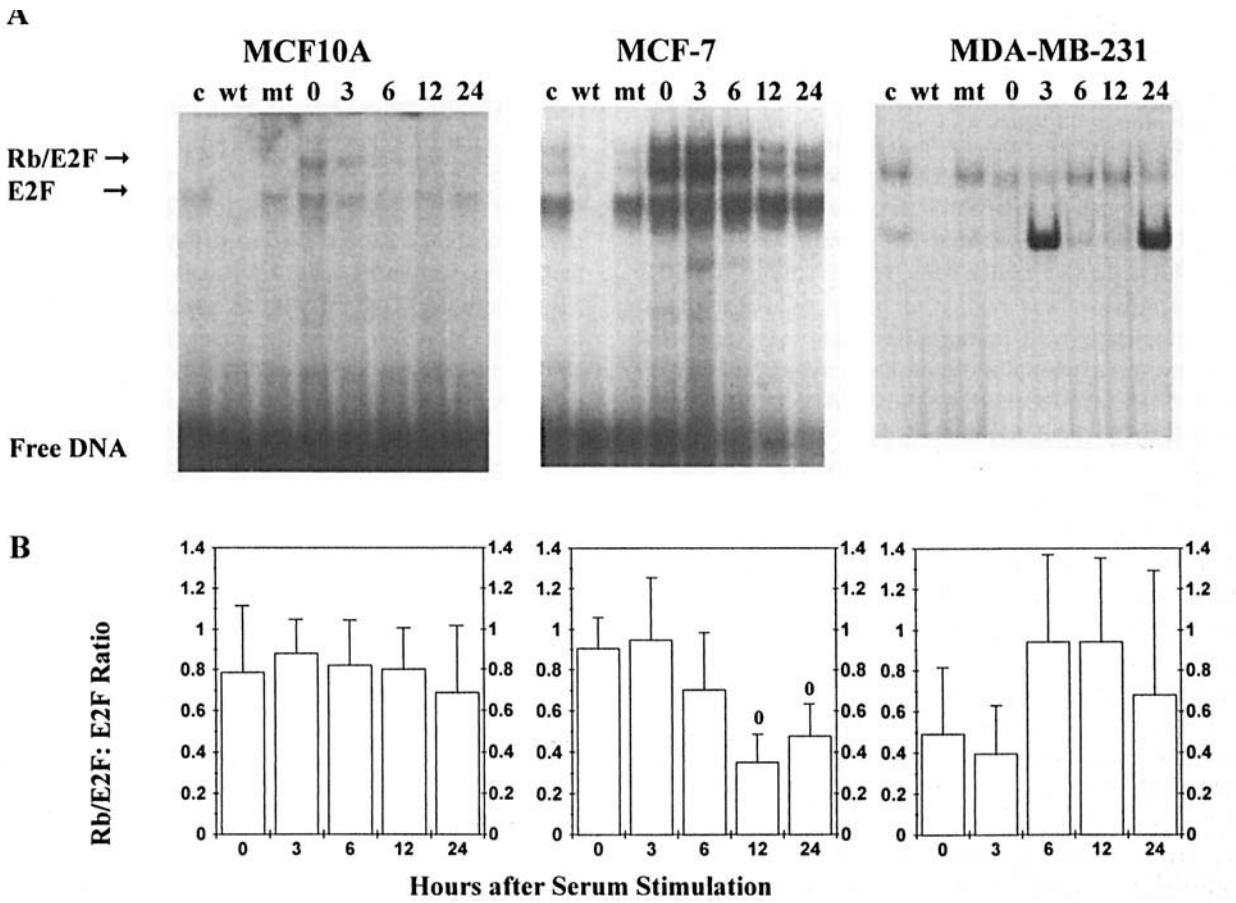

Figure 5. Autoradiographs and analysis of gel mobility shift assays using nuclear extracts from MCF10A, MCF-7, and MDA-231 cells and E2F promoter oligonucleotide. (A) Representative autoradiographs of gel shift assays. (C) Asynchronous positive control; wt, 50 molar excess of unlabeled E2F oligonucleotide; mt, 50 molar excess of unlabeled mutant oligonucleotide. Time (hr) after serum stimulation is indicated. E2F complexes are indicated with arrows. (B) Ratio of intensity of Rb/E2F band to intensity of the sum of the E2F bands versus time for three independent experiments as described in "Materials and Methods." Significant difference from 0 hr indicated with a "'0."'

cells) in each lane. This ratio, representing relative Rb activity, varied in each cell line with cell cycle progression. MCF10A cells had higher overall Rb activity than the tumor lines, and this activity decreased slightly (although not statistically significantly) with time (Fig. 5B). MCF-7 cells had high Rb activity at the early time points, followed by a significant decrease at 12 and 24 hr as compared with 0 hr (Fig. 5B). Although there were not statistically significant differences during the time course when all of the data for this cell line were combined, trends in Rb activity for MDA-MB-231 cells were consistently different from that of MCF10A and MCF-7 cells. As shown in Figure 5B, there was lower activity in MDA-MB-231 cells in the early time points (0 and 3 hr) as compared with MCF10A and MCF-7 cells. This was followed by higher Rb activity at 6 and 12 hr, dropping slightly at 24 hr. The overall Rb activity of the three cell lines was not significantly different in terms of amount of activity, but trends in Rb activity were unique in each cell type examined.

Cell-Cycle Regulated E2F Activity Consists of Multiple E2F Species. Although it has been reported that E2F-4 participates in the majority of E2F/DNA interactions in cell cultures (11, 40), E2F-1 has been identified as the most potent transcriptional activator of the E2F family (41). No significant supershift or immunodepletion of the Rb/E2F/DNA complex by the E2F-1 antibody was observed in MCF10A or MDA-MB-231 cells (Fig. 4, A, lanes 3, 5, 7, 9, and 12 and C, lanes 3, 5, 7, 10, and 12). Complete immunodepletion of the Rb/E2F/DNA complex by the E2F-1 antibody was observed at 3 hr in MCF-7 cells (Fig. 4B, fifth lane from the left). Immunoblotting nuclear protein from asynchronous cell populations with antibodies specific to E2F-1 and E2F-4 revealed that all three cell lines contained more E2F-4 than E2F-1 (data not shown). E2F4 antibody addition to the gel shift reaction slightly immunodepleted E2F/DNA complexes in MCF10A and MDA-MB-231 cells during early time points (data shown for 6 hr in MDA-MB-231 cells and 12 hr shown for MCF10A cells in Fig. 4, A and C), but the E2F4 antibody did not shift the Rb/E2F complex in these cells. In MCF-7 cells, the addition of the E2F-4 antibody resulted in a supershift of a higher molecular weight complex. This complex has been previously characterized in other cell types as the p107/E2F complex (42). As with MCF10A and MDA-MB-231 cells, the E2F4 antibody

did not appear to have any effect upon the Rb/E2F complex in MCF-7 cells (Fig. 4).

Discussion

Previous investigators have not quantitatively compared Rb expression between normal mammary epithelial cells and mammary adenocarcinoma cells. However, the qualitative impression was that Rb protein was generally lower in higher grade tumor cells. In our studies, total Rb protein expression was higher in breast adenocarcinoma cells as compared with immortalized mammary epithelial cells. Post-transcriptional mechanisms may account for the observed differences because we did not detect significantly higher cell cycle-regulated mRNA expression in the tumor cells. However, since fewer tumor cells synchronized in G1 as compared with MCF10A cells under the experimental conditions in which Rb protein levels were measured, Rb function was assessed by gel mobility shift assay. The results indicated that although the tumor cells had higher amounts of Rb protein, that protein did not exhibit consistent E2F binding activity when the cells were synchronized in G1 phase as it did in the MCF10A cells. These results suggest the importance of assessing Rb function rather than its presence in tumor cells as an indicator of prognosis in breast cancer.

Rb protein expression by immunoblotting has previously been examined in several breast cancer cell lines, revealing high variability in Rb protein levels in asynchronous cells (37). In this study, we have compared cell cycle-regulated Rb protein expression and found that overall, Rb protein was significantly higher in the tumor cells tested as compared with the immortalized mammary epithelial cells without significant differences in Rb mRNA levels. We hypothesize that post-transcriptional mechanisms may account for these differences, although Rb protein and mRNA half-life were not specifically examined. Rb protein has been shown to undergo estrogen receptor-dependent post-transcriptional modifications resulting in altered Rb protein expression in MCF-7 cells (18). In addition, the mechanism of Rb protein inactivation in cervical carcinoma cells by human papillomavirus (HPV) proteins has been intensively studied, and it is known to involve degradation by the ubiquitin proteosome pathway (43, 44). However, in other cell types, Rb is not known to be degraded by this pathway. Recently, two independent studies reported that the deubiquitinating enzyme Unp binds to Rb (45, 46). The function of this protein with respect to Rb degradation requires further investigation.

Comparison of Rb activity across the three cell lines revealed decreased Rb function in cells with characteristics of decreased state of differentiation and increased metastatic potential. MCF10A cells, representing immortalized mammary epithelium (19), had high Rb activity for the longest duration. MCF-7 cells, representing estrogen receptor-positive, non-metastatic breast adenocarcinoma cells (20, 47), had slightly lower Rb activity that significantly dropped with progression through the cell cycle. Poorly differentiated, highly metastatic MDA-MB-231 cells (21, 47) had abnormal cell cycle-regulated Rb activity. There was a delay in Rb activity in these cells as compared with MCF10A and MCF-7 cells.

The use of cell lines to study trends in biological activity of tumor cells is invaluable, especially when biochemical assays require the use of large quantities of cells that primary cultures cannot provide. In the case of breast adenocarcinomas, primary tumor samples are often difficult to obtain. However, over the years, several breast adenocarcinoma cell lines have been successfully established and have been well characterized. In many cases, the cultured cells do retain characteristics of breast adenocarcinoma and breast epithelium such as hormone receptor content, cytokeratin or vimentin expression, α-lactalbumin production, and metastatic potential (19–21, 48–51). The cell lines we chose to use are among those that have been well characterized. Each cell line was selected to represent a characteristic phenotype (e.g., high or low degree of differentiation and metastatic potential), however, this is a very small representation of breast adenocarcinoma *in vivo*. Our studies revealed low Rb function in poorly differentiated cells, which is consistent with studies showing loss of or lowered Rb expression in highly malignant breast adenocarcinoma primary tumors and cell lines. Rb activity as demonstrated in the gel shift assays is consistent with cell cycle-regulated changes in Rb phosphorylation in these cells as demonstrated by quantitative direct immunofluorescence studies using similar experimental conditions (Botos J, *et al.*, unpublished data).

Differences in cell cycle-regulated Rb activity between MCF10A and MCF-7 cells may result from two possible mechanisms. First, stimulation of estrogen receptors, which are not present in MCF10A or MDA-MB-231 cells (51), may increase the rate of cell cycle progression and Rb phosphorylation (52). Because phosphorylation inactivates Rb (53), estrogen-mediated increases in Rb phosphorylation could lead to decreased Rb activity. Although cells in the present study were not treated with estrogen, estrogen receptors may have been activated by phenol red and serum, both components of the media used to stimulate cells out of G0/G1 arrest. The other possible mechanism of lowered Rb activity in MCF-7 cells is Rb inactivation through cyclin E-regulated phosphorylation. Cyclin E expression is higher in MCF-7 cells than in MCF10A cells (22). Cyclin E, when complexed with cyclin-dependent kinase-2 (cdk2), has been found to phosphorylate Rb *in vitro* (54) and *in vivo* (53).

Abnormal Rb function in MDA-MB-231 cells is also possibly due to alterations in the dynamics of Rb phosphorylation. Expression of G1 cyclins (D1, D3, and E) is higher in MDA-MB-231 cells than in the other two cell lines (22). In addition, expression of p21, a G1 cdk inhibitor, is low (23). Together, these abnormalities could result in rapid Rb phosphorylation. Despite these possibilities, an increase rather than a decrease in Rb function was seen at 6 hr in

MDA-MB-231 cells. The increase in activity may result from increased dephosphorylation related to increased phosphatase activity. Specifically, cell cycle-regulated protein phosphatase activity should be determined in each cell type (55).

All three cell lines in this study lack p16 activity due to a mutation in the structural gene (23, 24, 37). The fact that there are detectable differences in cell cycle-regulated Rb activity across cell lines suggests that other factors are also important for maintaining Rb activity (p15, p18, p21, etc.) in mammary epithelium.

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