Microarray Analysis Reveals Glucocorticoid-Regulated Survival Genes That Are Associated With Inhibition of Apoptosis in Breast Epithelial Cells

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ABSTRACT

Activation of the glucocorticoid receptor (GR) results in diverse physiological effects depending on cell type. For example, glucocorticoids (GC) cause apoptosis in lymphocytes but can rescue mammary epithelial cells from growth factor withdrawal-induced death. However, the molecular mechanisms of GR-mediated survival remain poorly understood. In this study, a large-scale oligonucleotide screen of GR-regulated genes was performed. Several of the genes that were found to be induced 30 min after GR activation encode proteins that function in cell survival signaling pathways. We also demonstrate that dexamethasone pretreatment of breast cancer cell lines inhibits chemotherapy-induced apoptosis in a GR-dependent manner and is associated with the transcriptional induction of at least two genes identified in our screen, serum and GC-inducible protein kinase-1 (*SGK-1*) and mitogen-activated protein kinase phosphatase-1 (*MKP-1*). Furthermore, GC treatment alone or GC treatment followed by chemotherapy increases both SGK-1 and MKP-1 steady-state protein levels. In the absence of GC treatment, ectopic expression of SGK-1 or MKP-1 inhibits chemotherapy-induced apoptosis, suggesting a possible role for these proteins in GR-mediated survival. Moreover, specific inhibition of *SGK-1* or *MKP-1* induction by the introduction of *SGK-1*- or *MKP-1*-small interfering RNA reversed the antiapoptotic effects of GC treatment. Taken together, these data suggest that GR activation in breast cancer cells regulates survival signaling through direct transactivation of genes that encode proteins that decrease susceptibility to apoptosis. Given the widespread clinical administration of dexamethasone before chemotherapy, understanding GR-induced survival mechanisms is essential for achieving optimal therapeutic responses.

INTRODUCTION

Glucocorticoids (GCs) are essential steroid hormones required for the maintenance of several key physiological and developmental processes. GCs act by binding to the GC receptor (GR), which is followed by GR translocation into the nucleus and *trans*-activation or *trans*-repression of target genes (1). In addition, rapid "nongenomic" effects of GCs have been described (2). Although GCs induce apoptosis in lymphocytes (3), we (4) and others (5) have demonstrated that GCs can inhibit apoptosis in mammary epithelial cells (MECs). Similar findings have recently been reported in human and rat hepatocytes (6), vascular endothelial cells (7), and osteoclasts (8). Therefore, there appears to be a dual and cell-type-specific role for GCs in the regulation of cell death (9).

Synthetic GCs [*e.g.,* dexamethasone (Dex)] are often a component of chemotherapy regimens used for the treatment of lymphocytic malignancies (1). However, in breast epithelial tumors, GCs are administered as antiemetics and antihypersensitivity agents before treatment with doxorubicin and taxane-based chemotherapy regimens.

Remarkably, clinical studies have not addressed the potential effects of administering Dex before chemotherapy on tumor response. In view of the protective effects of GCs on growth factor deprivation-induced apoptosis (4), as well as a report of the inhibitory effect of GCs on paclitaxel-induced cell death in the breast tumor cell line Bcap37 (10), we further evaluated whether Dex pretreatment inhibits chemotherapy-induced cell death in the commonly used breast cancer cell lines MCF-7 and MDA-MB-231. At the same time that this manuscript was in preparation, Herr *et al.* (11) reported that Dex continuously added to animal drinking water inhibits the efficacy of cisplatin cytotoxicity in a lung cancer xenograft model. However, in these studies, direct transcriptional targets of GR activation involved in survival signaling were not identified, although differential effects on downstream caspases were observed in the GC treatment *versus* control groups.

To better understand the direct GR-mediated gene expression changes that might promote cell survival in MECs, we used high-density oligonucleotide microarrays to identify GC-regulated genes activated or repressed 30 min after Dex treatment. In MCF10A-Myc cells, we found that the expression of 30 genes was significantly down-regulated and that the expression of 45 genes was significantly up-regulated after Dex treatment. The majority of these target genes encode signal transduction proteins [*e.g.,* serum and GC-inducible kinase (SGK-1) and mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1)], metabolism-related proteins, putative transcription factors, and cell cycle/DNA repair proteins. Selected genes were verified by Northern blot analysis. We also show that GCs inhibit both paclitaxel and doxorubicin-induced apoptosis in the MCF-7 and MDA-MB-231 cell lines, suggesting a central role for the antiapoptotic effects of GR activation irrespective of chemotherapy type.

Two targets of GR activation were chosen for further study in this report. First, SGK-1, a downstream target of the phosphatidylinositol 3-kinase (PI3K) pathway, is a serine/threonine kinase that was shown previously to mitigate growth factor deprivation-induced apoptosis in neurons (12) and MECs (13). Second, MKP-1, a MAPK phosphatase, exhibits antiapoptotic effects in prostate cancer cells (14) and mouse fibroblast C3H10T1/2 cells (15). Overexpression of MKP-1 is also associated with increased tumorigenicity in breast (16), ovarian (17), and pancreatic cancers (18). We now show that endogenous SGK-1 and MKP-1 protein levels are increased in breast cancer cell lines treated with Dex before chemotherapy treatment. Furthermore, ectopic expression of either SGK-1 or MKP-1 inhibits chemotherapy-induced apoptosis to approximately the same degree as does Dex pretreatment. Using small interfering RNA (siRNA) to decrease SGK-1 and MKP-1 expression after GR activation, we also show a corresponding decrease in Dex-mediated protection from chemotherapy in the presence of either *SGK-1* siRNA or *MKP-1* siRNA. Taken together, these observations suggest that GR-mediated transcriptional activation of both *SGK-1* and *MKP-1* contribute to a GR-mediated signal transduction pathway that ultimately inhibits chemotherapy-induced apoptosis.

MATERIALS AND METHODS

Drugs. Paclitaxel (Calbiochem, La Jolla, CA) was dissolved in 100% methanol, and doxorubicin (Sigma, St. Louis, MO) was dissolved in 1×

phosphate-buffered saline (PBS) to make stock solutions, which were then diluted in culture medium to obtain the desired concentrations. Dex and RU486 (Sigma) were dissolved in 100% ethanol to make a stock solution of 10^{-3} M, which was then diluted in culture medium to obtain the desired concentration. The proteasome inhibitor, N-acetyl-Leu-Leu-norleucinal (ALLN; Sigma) was dissolved in 100% ethanol. A final concentration of 10 μM ALLN was used.

Cell Culture. The human MEC cell line, MCF10A-Myc, was used in the gene array analysis. MCF10A-Myc cells were cultured in a 1:1 mixture of DMEM and Ham's F12 (BioWhittaker, Walkersville, MD), supplemented with hydrocortisone (0.5 μg/ml), human recombinant epidermal growth factor (10 ng/ml), and insulin (5 μg/ml; Sigma). Cells were seeded into 10-cm dishes and, on reaching ~80% confluency, were incubated in serum-and growth factor-free medium for 72 h. Breast cancer cell lines were then stimulated for 30 min with vehicle alone (ethanol), Dex (10^{-6} M), or Dex/RU486 (10^{-7} M). All of the cells were grown in a humidified 5% CO_2 incubator at 37°C.

For chemotherapy experiments, the human MCF-7 and MDA-MB-231 breast cancer cell lines were obtained from American Type Culture Collection and grown in MEM or DMEM, respectively. For routine growth, media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were cultured for 16 h in serum-free medium and then were treated with vehicle (ethanol), Dex (10^{-6} M), or Dex/RU486 (10^{-7} M) for 1 h before paclitaxel (10^{-6} M) or doxorubicin (5×10^{-6} M).

Preparation of cRNA and Gene Chip Hybridization. Gene expression analysis was performed essentially as described in the Affymetrix Expression Analysis Technical Manual (19). Briefly, total RNA from MCF10A-Myc cells was extracted using Qiagen's RNeasy kit (Valencia, CA). Ten μg of total RNA were then used to synthesize double-stranded cDNA using the Superscript Choice System (Invitrogen, Grand Island, NY). First-strand cDNA synthesis was primed with a T7-(dT)$_{24}$ oligonucleotide. Three μg of log-phase gel-purified cDNA was used to synthesize biotin-labeled antisense cRNA using the BioArray High Yield RNA Transcript Labeling kit (Enzo Diagnostics, Farmingdale, NY). After precipitation and fragmentation, cRNA was hybridized to an Affymetrix HG-U95Av2 chip. The array was washed and stained with streptavidin phycoerythrin in a Fluidics Station 400 and then was scanned using an Affymetrix Gene Array Scanner. The entire experiment was performed three times.

Data Analysis. The image files from each experiment were processed using Gene Chip Analysis Software Suite 4.0. Global scaling to 2500 allowed normalization of data from different chips and absolute analyses were subsequently performed for each experiment. The scaled average difference value was calculated as the fluorescence intensity of mRNA expression between perfect match and central-mismatch oligonucleotide probe sets.[4] A second variation taken into account was the Affymetrix software call of "absent" (a gene intensity below an Affymetrix calculated threshold), "present," or "marginal" expression of a gene. Genes deemed absent in all three conditions in any one experiment were excluded from further analysis because these gene intensity values were below the threshold and are, therefore, considered to be unreliable. The third step was to allocate a value of 20 for gene intensities ≤20 because false positives may be more frequent in genes with very low levels of expression. The data generated from the above process was imported into GeneSpring 4.0 software (Silicon Genetics, Redwood City, CA) for selection of induced and repressed genes in each experiment.

We set a cutoff of ≥1.5-fold (Dex-treated *versus* vehicle-treated) for "induction" and ≤0.5 (Dex-treated *versus* vehicle-treated) for "repression" in all three experiments. Furthermore, genes induced (≥1.5-fold) by Dex but whose induction was reversed at least 20% by Dex/RU486 in each replicate experiment were considered likely targets of GR activation. Our goal was to identify GR-regulated genes whose expression was up-regulated by Dex but not by Dex/RU486 treatment. Because SGK-1 is a well-established direct transcriptional target of GR activation (13, 20), we used the minimal level of SGK-1 induction (1.5-fold) and the level of SGK-1 suppression of induction by RU486 (20%) as our cutoff for identifying genes of interest. In addition to the fold criteria specified above, comparisons between the Dex-treated and vehicle-treated gene expression levels, Dex/RU486-treated *versus* Dex-treated gene expression, across all three experiments were performed for each probe set using a paired *t* test on two degrees of freedom. Values of the *t* statistic

exceeding 4.30 or less than −4.30, corresponding to a two-sided *t* test at the 0.05 significance level, were regarded as evidence of significant induction or repression, respectively.

Northern Blot Analysis. For Northern blot analysis, 20 μg of total RNA were isolated from MCF10A-Myc cells that had been subjected to the same conditions as the microarray experiments and fractionated on a 1% agarose-formaldehyde gel and transferred to a nylon membrane. The following [^{32}P]dCTP-labeled cDNA probes were made using the Prime-It II Random Primer Labeling kit (Stratagene, Cedar Creek, TX): *MKP-1* [a gift from Dr. Christele Debros-Monbers, Institut National de la Santé et de la Recherche Médicale (INSERM), Paris, France], *IκB-α* (a gift from Dr. Guido Franzoso, University of Chicago, Chicago, IL), *GADD45α* (a gift from Dr. Dan Liebermann, Temple University, Philadelphia, PA) and rat *GAPDH* (13). Nylon membranes were hybridized overnight with the appropriate labeled probe. Membranes were then washed and exposed to film. Each experiment was performed at least two times.

Apoptosis Analysis. MCF-7 or MDA-MB-231 cells were trypsinized and seeded subconfluently at 1×10^5 cells/6-cm dish or on plastic chamber slides (Nalgene Nunc International, Naperville, IL). Cells were allowed to adhere overnight, were rinsed twice with 1× PBS, and were cultured for 16 h in serum-free medium. Vehicle (ethanol), Dex (10^{-6} M), or Dex/RU486 was then added to cells for 1 h before adding paclitaxel (10^{-6} M) for various time periods (8, 24, or 30 h). In some experiments, ALLN (10 μM) was added for 4 h before each collection time point. At each time point, cells were immediately fixed by adding formaldehyde at a final concentration of 7% to each well for 30 min. The fixative was aspirated, the wells were dried, and then cells were stained with a 1-μM 4,6-diamidino-2-phenylindole (DAPI)/1 × PBS solution as described previously (13). A Nikon Eclipse E800 microscope with UV illumination at ×600 was used to count at least 200 DAPI-stained cells in several fields to determine the percentage of apoptotic cells per experimental condition. All of the apoptosis assays were performed independently three times to calculate the average percentage of apoptosis and the SE. Statistical significance between two conditions in the apoptosis assays was determined by a one-sided Student's *t* test. A *P* <0.05 was considered significant.

Transfection. Transfections were performed using Effectene transfection reagent per manufacturer's instructions (Qiagen, Santa Clarita, CA). Briefly, MCF-7 cells or MDA-MB-231 cells were transfected with either the pLPCX or the pLPCX-encoding full-length SGK-1. Forty-eight h after transfection, transfectants were selected by exposure to 500 ng/ml puromycin. For transient transfections, MCF-7 cells or MDA-MB-231 cells were transfected with pLPCX (BD Bioscience, San Diego, CA), pFlagCMV2 (Sigma), pLPCX-HA-SGK-1 (13), or pFlagCMV2-MKP-1 (a gift from Dr. Andy Clark, Kennedy Institute, London, United Kingdom) or a combination of vectors. Forty-eight h later, cells were split into 6-well plates for apoptosis experiments and Western analysis.

Western Blot Analysis. Equal numbers of cells in each experimental condition were lysed with 2× Laemmli buffer and were fractionated on 10% SDS-PAGE gels (13). The fractionated proteins were transferred to nitrocellulose and were stained with Ponceau S dye to confirm equal protein loading. The membranes were then rinsed and incubated with anti-SGK-1 antibody (DB29, 1:1000 dilution, a rabbit polyclonal produced by immunization with a COOH-terminal SGK-1 peptide, Leu-Gly-Phe-Ser-Tyr-Ala-Pro-Pro-Thr-Asp-Ser-Phe-Leu-Cys) or with anti-MKP-1 rabbit polyclonal antibody (M-18, 1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). After washing with 1× Tris-buffered saline-Tween (0.1%), followed by incubation with peroxidase-conjugated goat antirabbit secondary antibody (1:5000 dilution), the membranes were treated with enhanced chemiluminescent staining (Amersham, Piscataway, NJ) per manufacturer's instructions before film development.

RNA Interference. The complementary siRNA sequences of individual target genes (sense shown in bold) were synthesized (Integrated DNA Technologies, Coralville, IA) with the following sequences: *SGK-1* forward oligonucleotide, 5′-**GTCCTTCTCAGCAAATCAA**TTCAAGAGATTGATTTG CTGAGAAGGACTTTTTT-3′; reverse oligonucleotide, 5′-AATTAAAA-AA**GTCCTTCTCAGCAAATCAA**TCTCTTGAATTGATTTGCTGAGAA-GGCC-3′, *MKP-1* forward oligonucleotide, 5′-**GGCAGACATCAGCTCC-TGG**TTCAAGAGACCAGGAGCTGATGTCTGCCTTTTTT-3′, reverse oligonucleotide, 5′-AATTAAAAAA**GGCAGACATCAGCTCCTGG**TCTC-TTGAACCAGGAGCTGATGTCTGCCGGCC-3′. A scrambled siRNA se-

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[4] The complete dataset for all three experiments can be viewed through the public user domain at http://madam.bsd.uchicago.edu:8080.

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quence was also generated as a control: control siRNA forward oligonucleotide, 5′-**TCACGCTACCTCATATACGCA**TTCAAGAGATGCGTATATG-AGGTAGCGTGATTTTTT-3′ and, reverse oligonucleotide, 5′-AATTAAA-AAA**TCACGCTACCTCATATACGCA**TCTCTTGAATGCGTATATGAG-GTAGCGTGAGGCC-3′. The *SGK-1* siRNA, *MKP-1* siRNA, or the control siRNA control target sequences as shown bold above were entered into the BLAST database, and no significant human homologies (except *SGK-1* for *SGK-1* siRNA, *MKP-1* for *MKP-1* siRNA) were detected. The complementary oligonucleotides for *SGK-1* siRNA, *MKP-1* siRNA or control siRNA were subcloned into the pSilencer1.0-U6 vector using *Apa*I and *Eco*RI (Ambion, Inc., Austin, TX). MDA-MB-231 breast cancer cells were transiently transfected with control siRNA, *SGK-1* siRNA, *MKP-1* siRNA, or both *SGK-1* and *MKP-1* siRNA plasmids. Forty h posttransfection, cells were split into chamber slides (Nalgene Nunc International) and were allowed to adhere overnight in 10% charcoal-stripped serum. The next day, either vehicle (ethanol) or Dex (10^{-6} M) was added for 1 h, followed by exposure to paclitaxel (10^{-6} M) for 8, 24, or 30 h. Analysis of the percentage of apoptotic cells and Western analysis to detect SGK-1 and MKP-1 protein expression was then performed as described above.

RESULTS

Genome-Wide Screen of GR-Mediated Gene Expression in MCF10A-Myc Cells. Three independent microarray experiments were performed in MCF10A-Myc cells to identify gene expression changes occurring 30 min after Dex treatment. Affymetrix 4.0 software calls a gene present, marginal, or absent depending on the intensity of normalized gene expression defined by a complex algorithm. Of the 12,696 genes on the HG-U95Av2 gene chip, an average $\pm$ SE of 6044 $\pm$ 397 genes (vehicle treatment), 5919 $\pm$ 151 genes (Dex treatment), and 6207 $\pm$ 193 genes (Dex/RU486 treatment) were called marginal or present. In the three experiments, an average of 13.2% of these marginal or present genes met the criteria for 50% down-regulation by Dex (1467 $\pm$ 144 genes/experiment), whereas an average of 9.7% of these genes met the criteria of $\geq$1.5-fold up-regulation by Dex (1090 $\pm$ 116 genes/experiment). However, using these fold criteria, only 69 down-regulated and 95 up-regulated genes were found to be common to all three experiments (Fig. 1*A*). To examine the consistency of gene regulation by Dex or Dex/RU486 across all three experiments, we performed a paired *t* test on the expression intensity after hormone treatment (compared with vehicle alone) for each gene. As Fig. 1*A* shows, we found that 45 genes of the 95 up-regulated genes and 30 of the 69 down-regulated genes had gene expression levels that were similar enough to yield a $P < 0.05$. These 75 up- and down-regulated genes could be further divided into five functional groups: signal transduction, metabolism, putative transcription factor, proapoptotic, or cell cycle/DNA repair genes (Fig. 1*B*). Conversely, 260 genes had significantly similar *P*s for expression across all 3 experiments, but did not meet the fold-change criteria of a 1.5-fold increase or a 50% decrease. This number reflects genes for which small differences in expression, although statistically consistent, were relatively insignificant in magnitude.

We also identified 34 putative direct GR target genes that were both induced $\geq$1.5-fold by Dex and also had their expression inhibited by at least 20% by the addition of the GR antagonist RU486 (Table 1). Of these 34 genes, 19 had consistent enough expression values for Dex up-regulation that the intensities across all three experiments achieved a *P* of <0.05. RU486 caused down-regulated gene intensities that were significantly consistent ($P < 0.05$) for 11 of these 34 genes. However, 29 of 34 genes had a *P* of <0.10 for up-regulation with Dex alone and 24 of 34 had a *P* of <0.10 for repression with RU486. Four of these likely GR target genes, *SGK-1*, *MKP-1*, inhibitor of nuclear factor κBα (*IκBα*) and growth arrest and DNA damage-inducible protein α (*GADD45α*), were further validated for gene
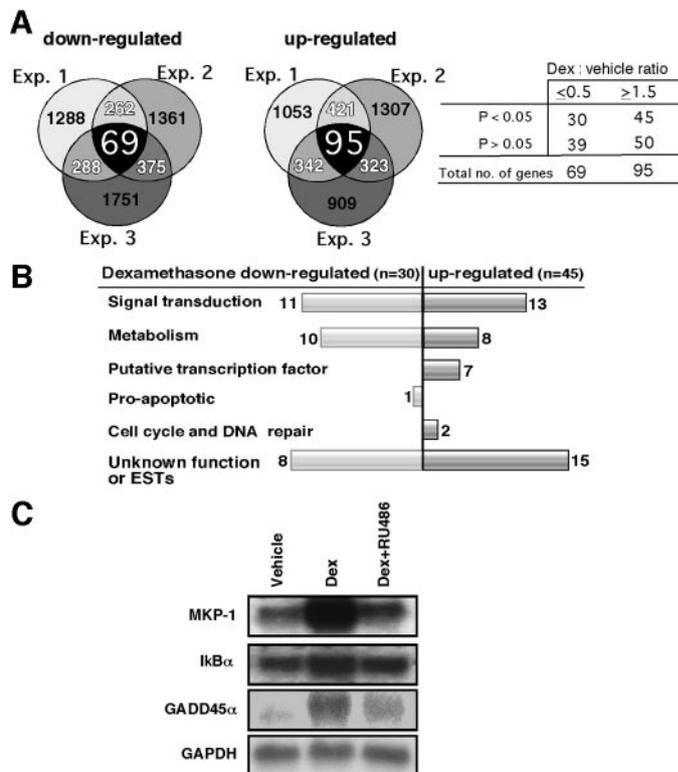

Fig. 1. Gene expression in MCF10A-Myc cells after dexamethasone (Dex) treatment. Total RNA was collected from MCF10A-Myc cells after treatment by vehicle alone, Dex (10^{-6} M), or Dex/RU486 (10^{-7} M). Isolated RNA from the three experimental conditions was then hybridized to the HG-U95Av2 Affymetrix chip, and expression data were analyzed as described in "Materials and Methods." *A*, Venn diagram showing the number of genes down- or up-regulated by Dex in each of three separate experiments (*Exp.*); *in the center,* the number of overlapping genes in all three experiments. *On the right, P* values for down- and up-regulated genes. *B*, functional clustering of Dex-regulated genes that meet both the fold change cutoff (down-regulation, $\leq$0.5; up-regulation, $\geq$1.5) and *P* <0.05. *EST,* expressed sequence tags. *C*, a subset of GR target genes identified from microarray experiments were evaluated by Northern analyses.

induction by Dex and inhibition by RU486 by Northern blot analysis (Ref. 13; Fig. 1*C*).

Unlike previous reports examining GC-induced expression of genes in hepatocytes (6), gastric cancer cells (21), and mouse MECs (5), no pro-survival Bcl-2 family members or inhibitors of apoptosis genes were identified by our genome-wide screen. Most of the proapoptotic genes including death ligands or receptors (*e.g., FasL/Fas*), caspases (*e.g., caspase-3, -8, -9*) or proapoptotic Bcl-2 family members were not identified as down-regulated by 30 min of Dex treatment in this rigorous screen. Interestingly, although the inhibitor of nuclear factor-kappa B (IκBα) was reliably up-regulated by Dex, known targets of NF-κB were not down-regulated in subsequent time course experiments, up to and including 24 h (data not shown). Taken together, gene expression profiling of MCF10A-Myc cells suggests that GR activation predominantly regulates signal transduction, metabolism, and transcription factor genes in epithelial cells rather than the cytokine and proapoptotic genes that have been identified in analogous experiments in lymphocytes (22, 23).

Dex Inhibits Chemotherapy-Induced Apoptosis in Breast Cancer Cells. Although nonmalignant MECs undergo apoptosis after growth factor withdrawal, we previously reported that many breast cancer cell lines do not die from prolonged serum withdrawal (24). To determine the role of GR activation in these growth factor-independent tumor cell lines, we induced cell death with chemotherapy and tested cells for the inhibition of apoptosis with Dex pretreatment. MCF-7 cells were pretreated with either Dex (10^{-6} M) or vehicle for

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Table 1 *Genes (n = 34) up-regulated by dexamethasone (Dex) and repressed by RU486 (RU) in three of three experiments*

GenBank accession no.	Gene product	Dex:Vehicle ratio[a]	P	Dex-RU:Vehicle ratio[a]	P[b]
Signal transduction					
S62539	Insulin receptor substrate 1 (*IRS-1*)	10.4 ± 2.9	0.02	2.5 ± 1.5	0.10
X70340	Transforming growth factor α (*TGFα*)	9.6 ± 6.0	0.06	3.1 ± 2.1	0.37
U07794	Tyrosine kinase (*TXK*)	9.5 ± 1.6	0.01	3.8 ± 2.8	0.14
M60047	Heparin-binding growth factor binding protein (*FGFBP1*)	9.1 ± 4.5	0.05	1.0 ± 0.0	0.05
X68277	CL 100/protein-tyrosine phosphatase (*MKP-1*)	6.4 ± 1.8	0.04	3.5 ± 1.4	0.08
X75342	*SHB* adaptor protein (a *Src* homology 2 protein)	6.3 ± 2.9	0.06	3.7 ± 2.0	0.13
U56387	Proprotein convertase subtilisin/kexin type 5 (*PCSK5*)	5.4 ± 2.3	0.08	2.0 ± 1.1	0.02
U47011	Fibroblast growth factor 8 (androgen-induced) (*AIGF*)	4.9 ± 2.4	0.10	0.4 ± 0.3	0.01
M22489	Bone morphogenetic protein 2 precursor (*BMP2A*)	3.6 ± 1.2	0.07	1.0 ± 0.4	0.15
Y10032	Serum and glucocorticoid regulated kinase (*SGK-1*)	3.3 ± 0.7	0.03	1.8 ± 0.3	0.02
M69043	IκB-α (*MAD-3/IκB-α*)	2.8 ± 0.4	0.02	1.4 ± 0.2	0.09
M60974	Growth arrest and DNA-damage-inducible, α (*GADD45α*)	2.1 ± 0.1	0.01	1.4 ± 0.1	0.01
Metabolism related					
X63359	UDP glycosyltransferase 2 family, polypeptide B10 (*UGT2*)	16.1 ± 7.9	0.05	2.2 ± 1.2	0.03
U37143	Cytochrome P450, subfamily (*CYP2J2*)	4.7 ± 0.4	0.03	1.7 ± 0.8	0.20
AJ000503	Dopachrome Δ-isomerase, tyrosine-related protein 2 (*TYRP2*)	4.0 ± 1.8	0.11	2.4 ± 1.4	0.06
L07765	Carboxylesterase 1 (monocyte/macrophage serine esterase 1 (*SES1*)	3.9 ± 2.2	0.19	2.7 ± 1.6	0.06
X78669	Calcium binding protein (*ERC55*)	1.9 ± 0.3	0.04	1.1 ± 0.1	0.08
Transcription					
U78722	Zinc finger protein 165 (*LD65*)	2.3 ± 0.2	0.01	0.4 ± 0.2	0.06
AF025770	Zinc finger protein 189 (*ZNF189*)	1.9 ± 0.2	0.04	0.7 ± 0.2	0.13
AF003540	Zinc finger protein 195 (*znfp104*)	1.8 ± 0.1	0.01	1.1 ± 0.2	0.09
X64318	Nuclear factor, interleukin 3 regulated (*NF-IL3A*)	1.6 ± 0.1	0.01	1.2 ± 0.1	0.01
Miscellaneous					
AJ01	Tropinin T1, skeletal, slow (ANM)	13.1 ± 6.2	0.09	1.0 ± 0.0	0.11
AJ003125	Cleavage of aminopropeptide of procollagens I, II and V (*PCPN1*)	8.7 ± 5.7	0.12	2.9 ± 2.3	0.14
AJ011733	Synaptogyrin 4 (*syngr4*)	3.1 ± 0.3	0.01	1.5 ± 0.3	0.01
Y12851	P2X7 receptor (*P2X7*)	2.4 ± 0.3	0.02	1.5 ± 0.1	0.02
AI865431	EST[c]	14.8 ± 1.9	0.01	1.1 ± 0.1	0.02
AI214965	EST	14.4 ± 4.7	0.01	3.7 ± 1.6	0.07
AL050379	EST	6.8 ± 2.9	0.11	3.2 ± 1.6	0.21
AA922934	EST	5.8 ± 2.9	0.09	0.9 ± 0.1	0.06
AL049437	EST	5.1 ± 0.5	0.01	1.2 ± 0.2	0.01
W27883	EST	5.0 ± 1.7	0.07	0.8 ± 0.3	0.12
AI688740	EST	4.9 ± 2.6	0.13	0.5 ± 0.3	0.001
AI635895	EST	3.3 ± 0.4	0.01	1.7 ± 0.3	0.06
AI991631	EST	1.9 ± 0.2	0.03	1.0 ± 0.1	0.06

[a] The mean ratio (derived from GeneSpring 4.0) of expression intensity ± SE in three independent experiments.
[b] Dex/RU486 *versus* Dex.
[c] EST, expressed sequence tag.

1 h and then treated with paclitaxel (10^{-6} M) alone for up to 30 h. This mimics the current schedule of Dex administration before chemotherapy in patients and also allows adequate time for GR-mediated gene expression before chemotherapy treatment in our *in vitro* model. Apoptosis was then measured by DAPI stain at 8, 24, and 30 h after chemotherapy. Fig. 2*A* shows that at 24 and 30 h, Dex pretreatment (*gray bars*) resulted in ~25% fewer apoptotic cells than paclitaxel-alone treated MCF-7 cells (*white bars*). Treatment with Dex alone consistently resulted in <5% apoptosis (Ref. 13 and data not shown). To determine whether Dex-induced inhibition of MCF-7 cell apoptosis is GR dependent, cells were pretreated with Dex and RU486 (10^{-7} M), a potent GR antagonist. Concurrent RU486 treatment reversed Dex-induced survival (Fig. 2*A, black bars*), suggesting a GR-dependent mechanism. Fig. 2*B* shows that Dex pretreated MDA-MB-231 cells (*gray bars*) also underwent significantly less apoptosis at 24 and 30 h after paclitaxel treatment ($P < 0.01$).

Doxorubicin is an anthracycline antibiotic with a wide range of clinical activity in solid and hematological malignancies. Several mechanisms appear to contribute to the cytotoxic effect of doxorubicin and related anthracyclines and include DNA intercalation, alkylation, and cross-linking; direct membrane effects; initiation of DNA damage via the inhibition of topoisomerase II; and free radical production with consequent DNA damage and lipid peroxidation (25). Paclitaxel, another commonly used effective chemotherapy for breast cancer treatment is believed to arrest cells in G_2-M phase by the stabilization of microtubules (26, 27). However, the exact mechanism by which paclitaxel induces apoptosis is not clear (28). Given the potentially different mechanisms of cytotoxicity between paclitaxel

and doxorubicin, we wanted to know whether Dex treatment before these two different chemotherapeutic agents would have a similar antiapoptotic effect. To evaluate this, we pretreated MCF-7 cells with or without Dex (10^{-6} M) before doxorubicin ($5 × 10^{-6}$ M) exposure, and we measured apoptotic cell death. As shown in Fig. 2*C*, MCF-7 cells treated with Dex before doxorubicin (*gray bars*) had significantly less apoptosis at 30 h compared with doxorubicin treatment alone (45.8 ± 3.8% *versus* 72.8% ± 3%; $P < 0.01$). Dex inhibition of doxorubicin-induced cytotoxicity was also examined in the estrogen receptor α-negative MDA-MB-231 cell line. As seen in Fig. 2*D*, Dex-pretreated MDA-MB-231 cells (*gray bars*) underwent significantly less apoptosis at 24 and 30 h after chemotherapy treatment compared with cells treated with doxorubicin alone ($P < 0.01$). Taken together, these results suggest that Dex pretreatment inhibits chemotherapy-induced apoptosis in breast cancer cells independent of cytotoxic mechanism.

SGK-1 and MKP-1 Steady-State Expression Levels Increase after GC Treatment. We have previously shown that ectopic expression of the GR target gene, *SGK-1*, inhibits growth factor deprivation-induced apoptosis (13). Unfortunately, in our previous studies we did not have an anti-SGK-1 antibody sensitive enough to detect endogenous SGK-1 and, therefore, we could only examine the antiapoptotic effects of ectopic SGK-1 protein expression. However, we now have an antibody that can detect endogenous SGK-1, and, therefore, we wanted to determine whether GC pretreatment leads to the induction of SGK-1 in breast tumor cell lines protected from chemotherapy-induced apoptosis. Fig. 3 shows a Western analysis of protein lysates from cells treated with Dex alone for 1 h, Dex followed by paclitaxel, or
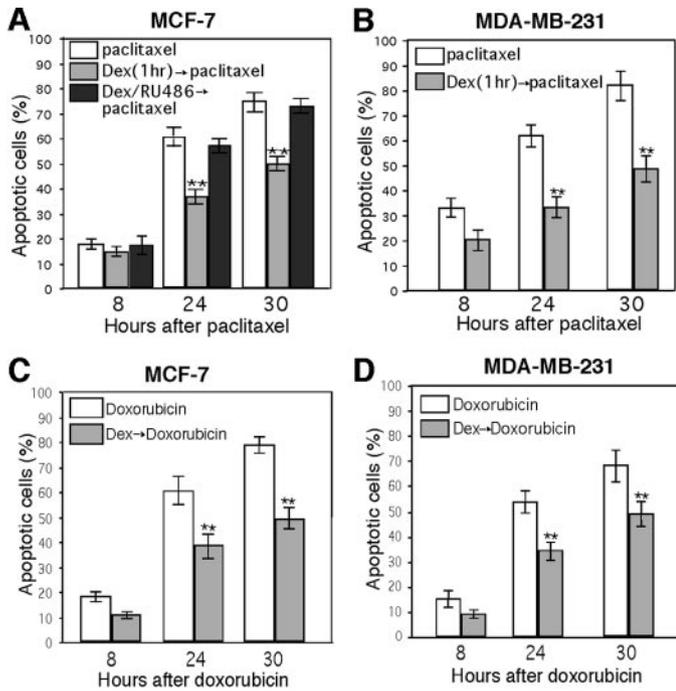
Fig. 2. Dex treatment inhibits chemotherapy-induced apoptosis in breast cancer cells. A, MCF-7 or B, MDA-MB-231 cells were treated with paclitaxel alone (10^{-6} M), dexamethasone (Dex; 10^{-6} M)/paclitaxel, or Dex/RU486 (10^{-7} M)/paclitaxel. Apoptosis assays were performed using 4,6-diamidino-2-phenylindole (DAPI) staining to score the percentage of apoptotic cells., C, MCF-7 or D, MDA-MB-231 cells were treated with either doxorubicin alone (5×10^{-6} M) or Dex/doxorubicin, and apoptosis assays were performed. All of the experiments were done in triplicate and the mean ± SE is shown. **, significantly less apoptosis ($P < 0.01$) in cells treated with Dex/chemotherapy compared with chemotherapy alone.

Expression of *MKP-1* has been reported to be associated with decreased apoptosis (14, 15) and increased tumorigenicity in several tumor cells (14, 16–18). Therefore, we examined MKP-1 protein expression in the same experiment. Fig. 3 shows that MKP-1 was induced in MDA-MB-231 cells by Dex or by Dex followed by paclitaxel, whereas MKP-1 was undetectable after treatment with either vehicle or paclitaxel alone. Paclitaxel alone had no effect on MKP-1 expression, suggesting that both SGK-1 and MKP-1 are specifically induced by Dex in this system. As with SGK-1, MKP-1 is ubiquitinated and degraded by the proteasome (30), and, consequently, steady-state MKP-1 levels increased dramatically after ALLN treatment. In MCF-7 cells, MKP-1 induction was detectable only in the presence of ALLN. Taken together, these results suggest that *SGK-1* and *MKP-1* are *bona fide* targets of GR activation in breast cancer cell lines.

Ectopic Expression of SGK-1 or MKP-1 Inhibits Chemotherapy-Induced Apoptosis. To determine whether SGK-1 or MKP-1 overexpression can mediate survival signaling independently of Dex treatment, MCF-7 and MDA-MB–231 cells were transfected with either pLPCX alone or pLPCX-HA-SGK-1. Cells were then selected in puromycin, and ectopic expression of HA-SGK-1 was confirmed by Western blotting (Fig. 4, *A* and *B*). Cells transfected with pLPCX or HA-SGK-1 were treated with chemotherapy for the indicated time periods, and apoptosis assays were performed. As shown in Fig. 4*A*, ectopic expression of SGK-1 in MCF-7 cells resulted in a significant decrease in paclitaxel- and doxorubicin-induced apoptosis at both 24 ($P < 0.05$) and 30 h ($P < 0.01$) after chemotherapy. In MDA-MB-231 cells (Fig. 4*B*), SGK-1 over-expression also decreased paclitaxel- (*gray bars*) and doxorubicin- (*black*
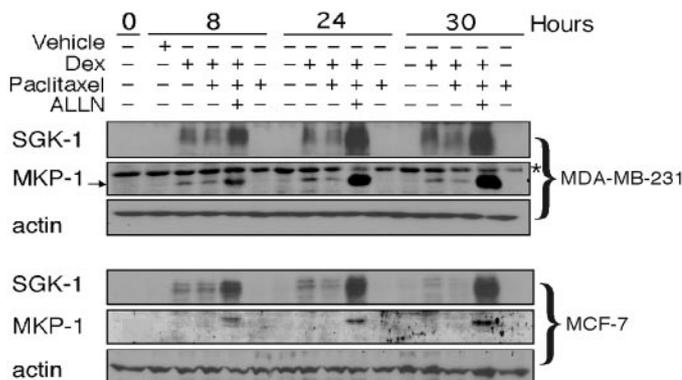

Fig. 3. SGK-1 and MKP-1 induction after dexamethasone (Dex) treatment in breast cancer cells. MDA-MB-231 or MCF-7 cells were treated as indicated. SGK-1 or MKP-1 protein expression was examined by Western blot analysis using either anti-SGK-1 (DB29) antibody or anti-MKP-1 (M18) antibody, respectively. Equal protein loading was confirmed by immunoblotting with an anti-β-actin antibody (actin). *, a cross-reacting, nonspecific band seen in MDA-MB-231 cells probed with the anti-MKP-1 antibody. ALLN, *N*-acetyl-Leu-Leu-norleucinal.
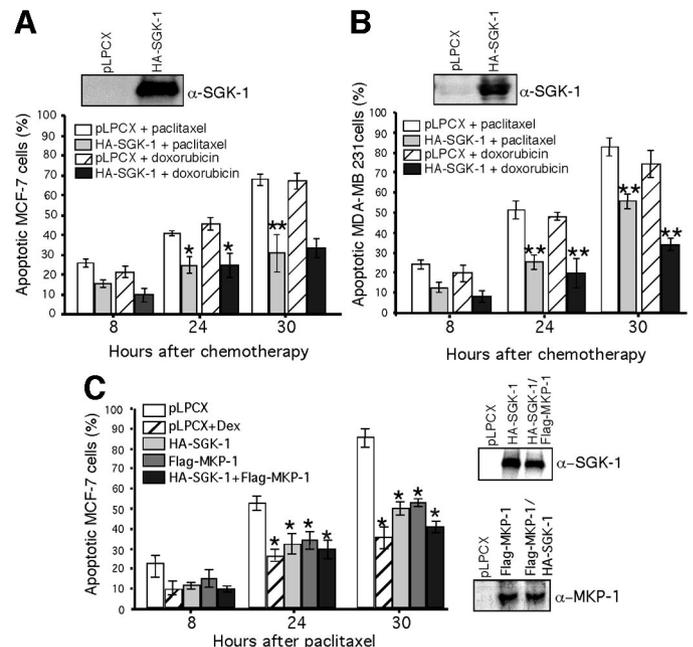

Fig. 4. Inhibition of chemotherapy-induced apoptosis in breast cancer cells after ectopic expression of SGK-1 or MKP-1. A, pLPCX-HA-SGK-1-transfected MCF-7 cell pools or B, MDA-MB-231 cell lines were examined by Western analysis for ectopic protein expression. Cells transfected with pLPCX or SGK-1 were treated with either paclitaxel (10^{-6} M) or doxorubicin (5×10^{-6} M) for the indicated times, and apoptosis assays were performed. All of the experiments were performed at least three times; the mean ± SE is shown. **, significantly less apoptosis ($P < 0.01$) in cells expressing ectopic SGK-1. C, MCF-7 cells transiently expressing pLPCX-HA-SGK-1, Flag-MKP-1, or both; SGK-1 and MKP-1 were treated with paclitaxel for the indicated times, and apoptosis was compared with parental cells transfected with the empty vector. All of the experiments were performed at least three times; the mean ± SE is shown. *, significantly less cell death ($P < 0.05$) in Dex-treated parental cells (*hatched bars*) and in cells ectopically expressing SGK-1 and/or MKP-1.

paclitaxel alone under the same conditions as those used in the apoptosis assays shown in Fig. 2. Baseline expression of SGK-1 (0 h time point) was undetectable, and vehicle treatment showed no induction of SGK-1 at any time point. However, the addition of Dex or of Dex followed by paclitaxel revealed an induction of endogenous SGK-1 protein in both MDA-MB-231 and MCF-7 cells. Consistent with the known rapid degradation of SGK-1 mediated by the proteasome (24), the induction of SGK-1 appeared to increase significantly by the addition of the proteasome inhibitor ALLN for 4 h before each time point. Paclitaxel treatment alone had no effect on SGK-1 expression in these cell lines, suggesting that increased SGK-1 expression is not induced simply as a stress response to apoptotic stimuli in mammary cells (29).
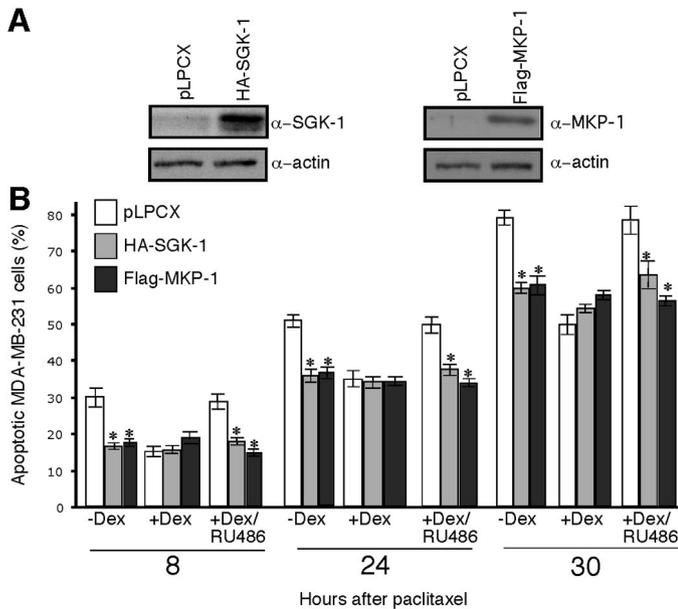
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Fig. 5. Ectopic expression of SGK-1 or MKP-1 does not augment dexamethasone (Dex)-induced survival. *A*, Western blots showing ectopic SGK-1 (*left panel*) or MKP-1 (*right panel*) expression in MDA-MB-231 cell lines. *B*, MDA-MB-231 cells, transiently expressing HA-SGK-1 or Flag-MKP-1, were treated with vehicle (−*Dex*), Dex (+*Dex*), or Dex/RU486 for 1 h before paclitaxel treatment, and then apoptosis was compared at the indicated times. ∗, significant protection in SGK-1 or MKP-1-expressing cells compared with vector-alone transfected cells ($P < 0.05$). There was no significant difference between vector alone, SGK-1, or MKP-1 expressing cells with Dex treatment.

bars) induced apoptosis. Taken together, these data suggest that SGK-1 overexpression is associated with the inhibition of chemotherapy-induced cell death, a finding that is consistent with the previous observations suggesting that ectopic expression of SGK-1 is associated with protection from growth factor deprivation-induced apoptosis in neurons (12) and MECs (13).

We next examined whether or not ectopic expression of a combination of GR-induced target genes might have additive or synergistic effects on inhibiting apoptosis. In these experiments, MCF-7 cells were transiently transfected with either pLPCX alone, HA-SGK-1, Flag-MKP-1, or both HA-SGK-1 and Flag-MKP-1. Forty-eight h after transfection, cells were treated with paclitaxel for 8, 24, and 30 h and were evaluated for apoptosis (Fig. 4*C*). Overexpression of SGK-1 (*light gray bars*), MKP-1 (*dark gray bars*), or both gene products (*black bars*) resulted in significantly less paclitaxel-induced apoptosis compared with cells expressing vector alone (*white bars*; $P < 0.05$). Moreover, ectopic expression of either SGK-1 or MKP-1 inhibited paclitaxel-induced apoptosis as efficiently as GC pretreatment (*hatched bars*), but expression of both genes did not exhibit synergistic protection. These data suggest that SGK-1 and MKP-1 up-regulation may contribute to the activation of a common GR-mediated survival signaling pathway that inhibits chemotherapy-induced apoptosis.

To determine whether ectopic expression of SGK-1 or MKP-1 provides additional protection beyond Dex-mediated antiapoptotic signaling, we transiently transfected MDA-MB-231 cells with either vector alone, HA-SGK-1, Flag-MKP-1, or both SGK-1 and MKP-1-expressing vectors. Forty-eight h later, transfected cells were treated with vehicle (ethanol), Dex, or Dex/RU486 for 1 h. Paclitaxel was then added for 8, 24, or 30 h and cells were scored for apoptosis. Fig. 5*A* is a Western blot showing that SGK-1 (*left panel*) or MKP-1 proteins (*right panel*) were efficiently expressed. In Fig. 5*B,* cells expressing either SGK-1 (*gray bars*) or MKP-1 (*black bars*) had significantly less apoptosis compared with cells expressing vector

alone (*white bars*; $P < 0.05$). However, the addition of Dex before chemotherapy reduced cell death significantly, and transient expression of either SGK-1 or MKP-1 did not increase survival above that afforded by Dex alone. Similarly, pretreatment of cells with concomitant Dex/RU486 reversed the protection by Dex alone, and transient expression of SGK-1 (*gray bars*), MKP-1 (*dark gray bars*), or both (data not shown) inhibited apoptosis similarly to that seen with Dex. Taken together, these data suggest that the transient expression of SGK-1, MKP-1, or both provides a survival signal in MDA-MB-231 cells that is similar in magnitude to that provided by GR activation.

Expression of *SGK-1* or *MKP-1* siRNA Reverses Dex-Induced Protection from Apoptosis. To determine whether specific inhibition of *SGK-1* and/or *MKP-1* expression can reduce the efficiency of Dex-induced survival signaling, we designed *SGK-1* and *MKP-1* siRNA molecules that prevent efficient induction of these protein products by Dex. Significant inhibition of both SGK-1 induction and MKP-1 induction were observed 24 h after Dex-treatment in cells expressing the specific siRNA molecules, but not in cells expressing control siRNA (Fig. 6*A*). Actin expression was not affected by Dex treatment, indicating specific regulation of the protein expression by RNA interference. Next, we sought to determine the effect of *SGK-1* siRNA or *MKP-1* siRNA on paclitaxel-induced apoptosis. Forty h post-siRNA transfection, cells were trypsinized, reseeded on plastic chamber slides overnight, and then treated with Dex/paclitaxel for various time periods. Apoptosis was evaluated by DAPI staining as shown in Fig. 6*B*, where apoptotic cells (*white arrows*) could be clearly distinguished from nonapoptotic cells (*black arrows*). Fig. 6*C* shows that *SGK-1* siRNA- (*gray bars*) or *MKP-1* siRNA- (*black bars*) expressing cells reversed the protection provided by Dex treatment before paclitaxel. For example, at 24 h after paclitaxel exposure, there was an average of 37% ± 6.5 apoptosis in *SGK-1* siRNA-expressing cells, and only 19.3% ± 0.58 apoptosis in control siRNA-expressing cells pretreated with Dex. At both 24 h and 30 h, expression of *SGK-1* or *MKP-1* siRNA led to similar levels of apoptosis in the presence of Dex that were equal to control cells not treated with Dex. These data suggest that Dex induction of either SGK-1 or MKP-1 is required for protection of chemotherapy-induced apoptosis in MDA-MB-231 cells.

DISCUSSION

GCs can elicit divergent biological outcomes through GR-mediated activation or repression of target genes. GC-induced cell survival appears to be common to many nonlymphocytic cell types (31). We previously observed that GC treatment inhibits both immortalized MECs and a subset of experimental breast cancer cell lines from growth factor withdrawal-induced apoptosis (13). To explore the underlying molecular mechanisms of GC-induced breast epithelial cell survival, we performed a genome-wide screen using high-density oligonucleotide microarrays to identify GC-regulated genes. Interestingly, the gene expression changes we observed in MCF10A-Myc cells differ significantly from those previously identified both in lymphocytes (32) and in the breast cancer tumor cell line T47D-GR (33). A possible explanation for these results is the differential expression of GR coactivator and corepressors in different cell types, a mechanism that has been proposed to explain the opposite effects of tamoxifen on mammary *versus* endometrial tissue (34). Some genes do overlap with those found in genome-wide screens of GR activation (35) in lymphocytes (*e.g., IκBα*); however, the induction of *IκBα* in MCF10A-Myc cells is of unclear functional consequence because known transcriptional targets of nuclear factor-kappa B activity do not
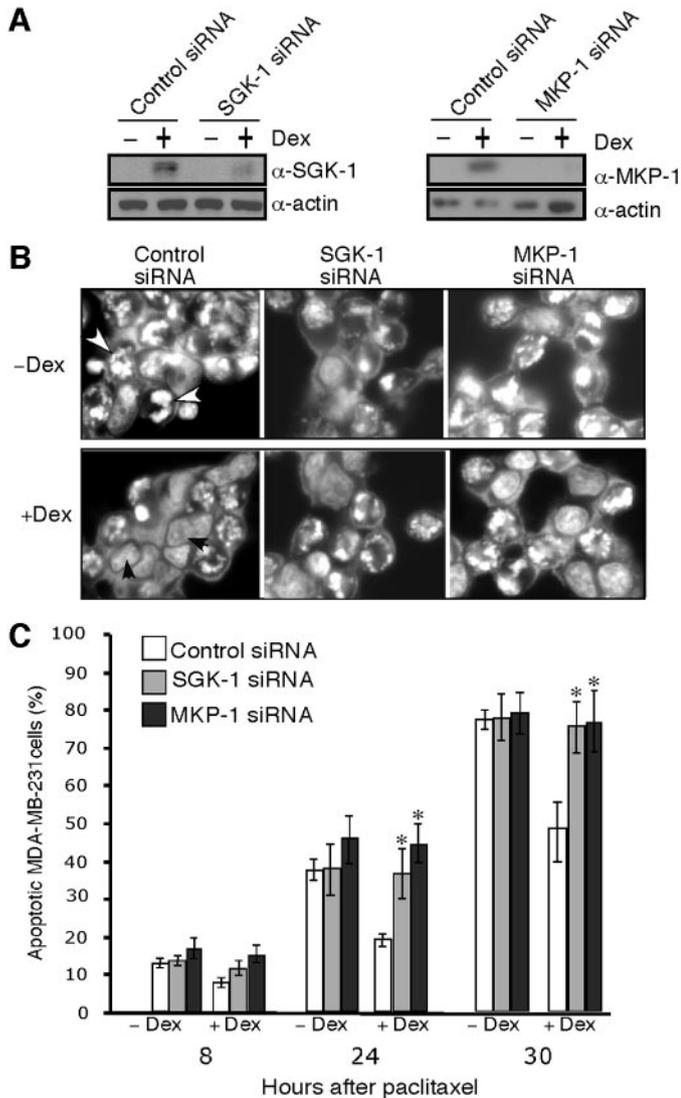
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Fig. 6. Expression of SGK-1 or MKP-1 small interfering RNA (siRNA) abolishes dexamethasone (Dex)-induced cell survival. *A,* Western analysis of MDA-MB-231 cells expressing SGK-1 siRNA (*left panel*) or MKP-1 siRNA (*right panel*). *B,* MDA-MB-231 cells expressing control siRNA (*left panel*), SGK-1 siRNA (*middle panel*) or MKP-1 siRNA (*right panel*) untreated (−*Dex, top row*) or Dex-treated (+*Dex,* 10^{-6} M; *bottom row*). *White arrows,* typically apoptotic cells; *black arrows,* nonapoptotic cells. *C,* average percentage of apoptosis in cells transiently expressing control siRNA, SGK-1 siRNA, or MKP-1 siRNA at indicated times after paclitaxel (10^{-6} M) treatment. All of the experiments were performed three times; the mean ± SE is shown. *∗,* significantly more cell death ($P < 0.05$) in Dex-treated cells ectopically expressing SGK-1 siRNA or MKP-1 siRNA compared with control siRNA-expressing cells.

appear to be repressed at subsequent microarray analysis time points.[5] Interestingly, we found that *MKP-1* is induced by Dex in MECs, although it appears to be down-regulated by Dex in pre-B-leukemic cells (35). The mechanism whereby the GR can activate or repress the same gene depending on cell type remains unknown.

Previous work has suggested that GC treatment can decrease chemotherapy-induced cytotoxicity in several tumor cell types. For example, concurrent GC and chemotherapy treatment has been shown to inhibit chemotherapy-induced apoptosis in Bcap37 breast cancer (10), HL-60 human leukemia (36), human glioma and rhabdomyosarcoma (37), human urothelial (38), and human cervical and lung cancer cell lines (11). However, in this study, we mimic the usual clinical

administration schedule of Dex before chemotherapy by treating cells 1 h before paclitaxel or doxorubicin. Furthermore, the inhibition of chemotherapy-induced apoptosis seems to be mediated through the activation of the GR because it can be blocked by concurrent treatment with the GR antagonist RU486 (Fig. 2*A*).

Little is known about the molecular mechanisms of the GC-mediated inhibition of chemotherapy-induced cell death. It is likely that cross-talk between the GR and a variety of signaling pathways may participate in cell survival. For example, Huang *et al.* (10) reported that Dex antagonizes paclitaxel-mediated nuclear factor-kappa B nuclear translocation and activation through induction of the IκBα protein and is associated with a decrease in paclitaxel-induced apoptosis in the Bcap37 breast cancer cell line. Here, we provide additional evidence that both SGK-1 and MKP-1 proteins are up-regulated after GC pretreatment in breast cancer cells and that ectopic expression is associated with the inhibition of chemotherapy-induced apoptosis in breast cancer cells. Furthermore, *SGK-1* and *MKP-1* siRNA each decreased protein expression and subsequently reversed Dex-induced survival (Fig. 6*C*).

SGK-1 is a protein kinase A, B, G, C family member that has been shown to contribute to the inactivation of the proapoptotic forkhead transcription factor FKHRL1 (12). In addition, SGK-1 has been shown to phosphorylate and inactivate B-Raf, which is upstream of extracellular signal-regulated kinase (ERK) phosphorylation (39). One study reported that paclitaxel-induced cell death may require ERK phosphorylation (40) because pretreatment with the ERK inhibitor, PD 98059, could reverse paclitaxel-induced MCF-7 cell apoptosis. This observation is in contrast to more recent reports that ERK inhibitor treatment can potentiate chemotherapy toxicity in other cell types, although the timing of ERK inhibitor administration relative to chemotherapy treatment may determine the specific outcome (41, 42). In addition to the induction of MKP-1, it has also been reported that GR may cross-talk with the MAPK signal pathway through directly or indirectly interacting with Raf-1, which in turn prevents its activation (43). These studies suggest that GCs can cause MAPK dephosphorylation through multiple pathways. We, therefore, hypothesize that *SGK-1* and *MKP-1*, two GR target genes, may act in concert to acutely inhibit MAPK phosphorylation, thereby decreasing the efficiency of chemotherapy-induced cell death. This hypothesis is the subject of ongoing studies examining the role of SGK-1 and MKP-1 in inhibiting common MAP kinase signaling pathways.

In summary, we have used large-scale oligonucleotide microarrays to identify GR-regulated genes in MECs. Perhaps surprisingly, several of the genes identified are signaling molecules (kinases and phosphatases) and transcription factors, and only a few of the genes that we identified in MECs are the same as those identified previously as GC-regulated in lymphocytes. This suggests that tissue-specific differences in GC-induced apoptosis *versus* survival outcomes may be due to cell-type-specific transcriptional regulation. These results also link GR activation to both the PI3K/SGK-1 and the MAPK signaling pathways. Understanding how GCs inhibit cell death may lead to the identification of molecular targets for cancer treatment. Finally, the widespread use of GCs before chemotherapy requires reevaluation because of the observed inhibition of chemotherapy efficacy seen in this and other studies.

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[5] W. Wu and S. D. Conzen. Gene Profiling Following Glucocorticoid Receptor Activation in Mammary Epithelial Cells, manuscript in preparation.

microarray database. We also thank Gini Fleming and Geof Greene for useful discussions.

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Microarray Analysis Reveals Glucocorticoid-Regulated Survival Genes That Are Associated With Inhibition of Apoptosis in Breast Epithelial Cells

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