Glucocorticoid Receptor-mediated Protection from Apoptosis Is Associated with Induction of the Serine/Threonine Survival Kinase Gene, sgk-1*

We previously demonstrated that activation of the glucocorticoid receptor (GR) initiates an antiapoptotic signal in the immortalized human mammary epithelial cell line MCF10A that is dependent on the GR's transcriptional activity. In this study, we show that the survival role of GR activation extends to protecting human breast cancer cells undergoing apoptosis after growth factor deprivation. Serum and glucocorticoid-regulated kinase-1 (sgk), a gene previously identified as a direct transcriptional target of the activated GR in a rat mammary tumor cell line, was rapidly induced after GR activation in human mammary epithelial cells. Furthermore, in the absence of all growth factors, ectopic sgk expression inhibited apoptosis, suggesting that SGK is a survival kinase. Finally, kinase-dead SGK expression inhibited the protection from apoptosis usually seen after GR activation. These findings suggest that SGK is an important downstream target of GR-mediated survival signaling and that it is distinct from other survival kinases because it can be primarily regulated at the level of transcription.

The specificity of the glucocorticoid receptor's (GR) transcriptional activity varies widely between cell types, thus accounting for the diverse and sometimes opposite physiological effects of glucocorticoid in different tissues. For example, glucocorticoids have been shown to promote apoptosis in lymphocytes (1, 2), whereas human mammary epithelial cells (MECs) (3) and rat hepatoma epithelial cells (4) are protected from apoptosis after GR activation. Furthermore, studies in both breast and liver epithelial cells have demonstrated that RU486, a potent GR antagonist that inhibits GR-mediated transcriptional activation, reverses the survival effect of glucocorticoids (3, 4). The antagonistic effects of RU486 on cell survival suggest that glucocorticoid-mediated survival is regulated specifically through GR-induced transactivation of downstream genes.

Previous studies using glucocorticoid concomitantly with or without the GR antagonist RU486 have suggested that the identification of genes directly induced or repressed by GR activation might reveal important pathways relevant to epithelial cell survival signaling. One such GR-inducible gene, serum and glucocorticoid-regulated kinase-1 (sgk), encodes a serine/threonine kinase with 54% homology in its catalytic domain to the well described antiapoptotic kinase AKT. sgk was originally identified through subtractive cloning of a serum and glucocorticoid-induced mammary tumor cell cDNA library (5). More recently, sgk was found to be part of a larger gene family and was designated sgk-1 (6). Interestingly, sgk has also been shown to be transcriptionally induced after activation of a variety of steroid receptors including the mineralocorticoid receptor in kidney epithelial cells (7) and the follicle-stimulating hormone receptor in ovarian granulosa cells (8). sgk transcripts have also been shown to be induced after changes in cell volume (9) and under conditions of extracellular hyperosmotic stress (10).

In this report, we have extended our original investigation of GR-mediated survival signaling from the nontumorigenic MEC cell line MCF10A to breast cancer cell lines. Although only a subset of commonly studied human breast tumor cell lines undergo significant apoptosis after growth factor deprivation, most of these growth factor-dependent cell lines were protected from apoptosis after treatment with physiological concentrations of glucocorticoid. Furthermore, the effect of glucocorticoid appears to be GR-mediated because it can be out-competed by high affinity GR antagonists. We also demonstrate that the GR survival signal is likely to be transmitted at least in part as a consequence of the transcriptional activation of sgk since (i) sgk mRNA is induced in a GR-dependent fashion immediately after glucocorticoid treatment of human MECs, (ii) ectopic SGK expression protects MECs from apoptosis induced by growth factor withdrawal, and (iii) expression of a kinase-dead SGK inhibits protection from apoptosis. Taken together, these results suggest the existence of a novel GR-initiated antiapoptotic pathway that operates, at least in part, through transcriptional induction of the survival kinase gene, sgk.

MATERIALS AND METHODS

cDNA Constructs—POG-hSGK was obtained as a generous gift from Dr. Siegfried Waldegger (University of Hamburg, Germany). The human sgk cDNA fragment was amplified from POG and cloned into the EcoRI and XhoI sites of the retroviral vector pLPCX (CLONTECH, Palo Alto, CA) by applying a polymerase chain reaction-based strategy that incorporated a hemagglutinin (HA) tag in-frame in the amino terminus of wild type SGK using the following primer (Life Technologies, Inc.): wild type HA-SGK, 5'-TAATACGACTCACTATAGGCCCATGCTAGCCATGTTCCAGACTGCTAGGTTGAAAAACT-3'. AN60 HA-SGK was
Similarly constructed by inserting an HA tag in-frame 5' to the coding sequence for amino acid 61 of SGK: ΔN60 HA-SGK, 5'-GGAATTCCTCAGAGGAAAGATGTCACACATG-3'. The carboxyl-terminal primer for both constructs was 5'-GGAAATTCCTTGAGGGGATCTGTCACACATG-3'. The polymerase chain reaction mixture was supplemented with 10% Me2SO and run with Pho-Δlac vectors (Promega, Madison, WI), with 95 °C, 5 min denaturation followed by 35 cycles at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min, and a final 72 °C for 5 min elongation. Mutations were confirmed using primers provided with the pLPXC vector by overlapping bidirectional sequencing using an ABI77 sequencing kit (PerkinElmer Life Sciences).

Cell Culture and Viral Infections—Whole cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). MCF10A and MCF10A-Myc cells (as described in Moran et al. (3)) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (BioWhittaker, Walkersville, MD) supplemented with hydrocortisone (0.5 μM), human recombinant EGF (10 ng/ml), and bovine insulin (5 μg/ml, Sigma). BT-20, H-S78T, MDA-MB-231, MDA-MB-468, SK-BR-3, and T-47D cells were cultured in Dulbecco's modified Eagle's medium (BioWhittaker) supplemented with 10% heat-inactivated fetal calf serum (FCS; Atlanta Biologicals, Norcross, GA). MCF7 cells were cultured in minimal essential medium (ATCC) supplemented with 10% heat-inactivated FCS, and HCC1937 cells were cultured in RPMI 1640 (BioWhittaker) supplemented with 10% heat-inactivated FCS. All medium were made by direct transfection of retroviral vectors into amphotrophic Phoenix cells (a gift of Dr. Gary Nolan, Stanford University, Palo Alto, CA) using either standard calcium phosphate precipitation or Effectene transfection reagent per manufacturer's instructions (Qiagen, Santa Clarita, CA). MECs were infected as described previously (3) with HA-SGK-expressing retroviruses, and clones were selected with puromycin (400 ng/ml). Individual colonies were tested for HA-SGK expression by Western analysis using an anti-HA monoclonal antibody.

Apoptosis Assays—Cells were trypsinized and seeded subconfluently at 1 × 10^5 cells/cm² well in the appropriate medium. Cells were allowed to adhere overnight, rinsed twice with PBS, and subsequently cultured for 72 h in serum-free media containing insulin, EGF, hydrocortisone, dexamethasone, 17α,20β-dihydroxy-4-androstene-3,17-dione, 17α,20β-dihydroxyprogesterone, and dexamethasone oxetanone (10⁻⁶ M; Sigma), or combinations of these purified growth factors in the concentrations listed above. In GR antagonist assays, RU486 (5 × 10⁻⁹ M; Sigma), dexamethasone 21-mesylate (DM, 10⁻⁷ M; Steraloids Inc, Newport, RI), and dexamethasone oxetanone (10⁻⁸ M; a gift of Dr. Stoney Simons, National Institutes of Health) were added to media containing glucocorticoid.

In some experiments, cells were pre-treated for 30 min with serum-free media and 50 μM LY294002 (Calbiochem) or vehicle alone (0.01% Me2SO in PBS) before the addition of appropriate growth factors. After a 72-h incubation, media and floating debris were gently aspirated from wells and replaced with 2 ml of fresh serum-free media; cells were allowed to grow in the appropriate media. When cells reached ~80% confluency, media was aspirated, and cells were washed twice with PBS and incubated in serum-deprived (0.5% FCS) media for 72 or 96 h. After serum deprivation, cells were stimulated for 30 min with either vehicle alone (ETOH), dexamethasone, dexamethasone/RU486, or 20% FCS. Total RNA was harvested using the RNeasy mini kit per manufacturer's instructions (Qiagen). RNA was quantified by spectrophotometry, and 20 μg of RNA per experimental group was electrophoresed in a 1.0% agarose, 17% formaldehyde gel and subsequently transferred to a nylon membrane (Amersham, Pharmacia Biotech). Membranes were then sequentially hybridized with a full-length human sgk cDNA probe and a rat gapdh cDNA probe labeled with [α-³²P]dCTP using the Prime-It II random primer labeling kit (Stratagene, Cedar Creek, TX). Membranes were then washed and exposed to film. Bands were quantified using a Bio-Rad GS-710 calibrated imaging densitometer so that the relative ratio of sgk/gapdh signal could be determined. Experiments were repeated at least two times to calculate average ratios of sgk/gapdh mRNA intensities and S.E.

Site-directed Mutagenesis—The kinase-dead mutation (K127M HA-SGK) and the phosphorylation site mutations (T256A and S422A HA-SGK) of HA-tagged hSGK were made using the QuikChange site-directed mutagenesis kit (Stratagene) per the manufacturer's instructions. Primers were synthesized to alter either the hSGK ATP-binding site, lysine 127, to methionine or the putative phosphorylation sites threonine 256 or serine 422 of hSGK to alanine. Primers were synthesized to alter either the hSGK ATP-binding site, lysine 127, to methionine or the putative phosphorylation sites threonine 256 or serine 422 of hSGK to alanine. Primers (Life Technologies, Inc.) consisted of the following sequences: K127M HA-SGK, 5'-TCTTACGCTGCTTGTGGTTTAAAGAAGAAACATC-3'; T256A HA-SGK, 5'-CACACAGCACAACATCCGATCTGTTGCACGCCGGGAC-3'; S422A HA-SGK, 5'-GCGAGGCTTTCCTGGGCTTTG-CACAACAGCACAACATCCGCATTCTGTGGCAC-3'; 5'-GCCGAGGCTTTCCTGGGCTTTG-CACAACAGCACAACATCCGCATTCTGTGGCAC-3' (the mutated codons are underlined). The template DNA used was HA-tagged pLPXC-hSGK. Mutations were confirmed using primers provided with the pLPXC vector by bidirectional sequencing using an ABI77 sequencing kit (PerkinElmer Life Sciences). Mutant pLPXC-hSGK vectors were then used to generate retroviruses and infect MECs as described above.

RESULTS

Glucocorticoids Inhibit Apoptosis of Human Breast Cancer Cells via Activation of the GR—We previously demonstrated that GR activation initiates a potent antiapoptotic signal in the nontumorigenic MEC line MCF10A and its derivative line MCF10A-Myc. In the current study, we wished to determine whether GR activation might also inhibit apoptosis in breast cancer cell lines. We therefore selected eight commonly studied breast cancer cell lines (BT-20, HCC1937, H-S78T, MCF7, MDA-MB-231, MDA-MB-468, SK-BR-3, and T-47D) and evaluated them for GR expression. Western blot analysis of whole cell extracts using affinity-purified anti-human GR antibodies revealed that all eight cell lines expressed the GR protein (Fig. 1A). Although as previously reported, the T-47D line had relatively little GR expression (Fig. 1A). To determine whether glucocorticoid treatment can mediate a survival pathway in these tumor cell lines, apoptosis was measured after 72 h in the absence of all growth factors (including glucocorticoid) and in the presence of a physiological concentration (10⁻⁶ M) of the synthetic glucocorticoid dexamethasone. In the absence of all growth factors, the average percentage of apoptosis in breast cancer cell lines varied from 10.6% in MDA-
MB-231 cells to only 0.6% in MCF7 cells (Fig. 1B). In four of the eight tumor cell lines tested, MDA-MB-231, BT-20, Hs578T, and MDA-MB-468, treatment with dexamethasone significantly inhibited apoptosis when compared with complete growth factor deprivation (p < 0.05).

To determine whether glucocorticoid-mediated survival was functioning specifically through activation of the GR, MCF10A-Myc and MDA-MB-231 cells were treated concomitantly with dexamethasone and one of three known antagonists of GR activation and its subsequent transcriptional activity: RU486 (5 × 10⁻⁵ M) (16), dexamethasone oxetanone (10⁻⁵ M) (17), or dexamethasone 21-mesy late (10⁻⁷ M) (18). All three GR antagonists reversed the antiapoptotic effect of glucocorticoids significantly (p < 0.05), although to varying degrees depending on the cell line examined (Fig. 2B). In MCF10A-Myc cells, RU486 co-treatment was the most potent antagonist of glucocorticoid-mediated survival, resulting in a 4.5-fold increase in apoptosis. In MDA-MB-231 cells, however, DM was the most potent antagonist of survival signaling by glucocorticoids. Therefore, we first confirmed our array data using Northern analysis of GR expression in MCF10A-Myc cells and a panel of breast cancer cell lines.

**Transcriptional Induction of the Immediate Early Response**

As expected for a receptor-mediated mechanism of glucocorticoid activation, the effect of all three GR antagonists could be reversed by GR activation and its subsequent transcriptional activity: RU486 (data not shown). One of the most intriguing GR-induced genes identified from this differential display of MEC transcripts was sgk, encoding a putative serine/threonine kinase previously shown to be 54% homologous in its catalytic domain to the well characterized antiapoptotic serine/threonine kinase AKT (5). Although sgk had been shown to be transcriptionally induced by glucocorticoid treatment in rat mammary carcinoma cells (5), another study found that sgk transcripts were not induced by glucocorticoid treatment in human kidney epithelial cells (9). This raised the possibility of a species-specific difference in sgk promoter activation by glucocorticoids. Therefore, we first confirmed our array data using Northern analysis of sgk mRNA transcripts expressed 30 min after glucocorticoid treatment in two glucocorticoid-sensitive human breast cell lines, MCF10A-Myc and MDA-MB-231. Northern blot analysis of total cellular mRNA using an α-³²P-labeled full-length human sgk cDNA probe demonstrated that in MCF10A-Myc cells, GR activation significantly induced sgk mRNA (Fig. 3A). The GR antagonist RU486 inhibited sgk mRNA induction, whereas gapdh transcript expression remained relatively constant under all conditions. MDA-MB-231 cells had a similar, although slightly less robust, induction of
Ectopic Overexpression of Wild Type sgk Inhibits Apoptosis—We next investigated the biological activity of ectopically expressed SGK in MCF10A-Myc cells subjected to apoptotic stress. SGK was ectopically expressed in MCF10A-Myc cells after transduction with retroviruses encoding either an HA-tagged wild type human SGK (HA-SGK) or a truncated HA-SGK containing amino acids 61–431 (tagged wild type human SGK (HA-SGK) or a truncated HA-SGK). The truncated ΔN60 SGK protein had been previously shown to be more efficiently expressed than wild type SGK in human embryonic kidney 293 cells (6). Individual clones expressing either wild type HA-SGK or ΔN60 HA-SGK were isolated by puromycin selection, and whole cell lysates were examined for HA-SGK protein expression by Western analysis using a monoclonal anti-HA antibody. Similar to previously reported results in human embryonic kidney 293 cells, ΔN60 HA-SGK was much more efficiently expressed than the full-length, wild type HA-SGK, although both were clearly visible in comparison to cells transduced with the empty retroviral pLPCX vector alone (Fig. 4A). To determine whether wild type and ΔN60 HA-SGK have kinase activity when overexpressed, MCF10A-Myc cells stably expressing these two constructs were deprived of all growth factors overnight, and SGK activity was measured. Fig. 4B presents the kinase activity of SGK immunoprecipitated from wild type HA-SGK, ΔN60 HA-SGK, and a kinase-dead K127M HA-SGK expressed in MCF10A-Myc cells. Since an efficient substrate for SGK kinase activity is not known, we used the previously described SGK-tide as a substrate (12). Elevated kinase activity was seen in both wild type and ΔN60 HA-SGK immunoprecipitated from MCF10A-Myc cells when compared with K127M HA-SGK. Immunoprecipitated wild type and ΔN60 HA-SGK both appeared as a doublet in Western analysis, suggesting that phosphorylated forms of SGK exist in MCF10A-Myc cells despite the absence of growth factors in the culture medium.

SGK-overexpressing MCF10A-Myc cell lines were then evaluated for apoptosis after various conditions of growth factor deprivation. In the absence of all growth factors (Fig. 4C), both wild type and ΔN60 HA-SGK-expressing cell lines showed an average of ~60% inhibition of apoptosis compared with control cells expressing only the empty pLPCX vector. These results suggest that even in the absence of growth factors, expression of either the wild type or truncated ΔN60 HA-SGK can protect MECs from apoptosis. Interestingly, the addition of insulin and EGF (shown previously in MCF10A cells to stimulate the PI 3-kinase pathway sufficiently to induce AKT phosphorylation (3)) did not significantly increase survival in cells expressing...
ectopic mutant HA-SGK expression in MCF10A-Myc cells. S.E.

We next asked whether kinase-dead SGK could act as a functional dominant negative construct and inhibit survival in MECs treated with glucocorticoid. Cell lines overexpressing K127M HA-SGK were deprived of all growth factors or treated with dexamethasone (10^{-6} M) alone for 72 h. In K127M HA-SGK-expressing cell lines treated with glucocorticoid, a small but consistent increase in apoptosis was seen when compared with the parental cell line (Fig. 5C). These results suggest that the survival effect seen after glucocorticoid induction of SGK can be partially abrogated by the presence of kinase-dead SGK, implying a possible dominant negative function for kinase-dead SGK.

The PI 3-Kinase Pathway Is Required for SGK Survival

We have identified a novel pathway of mammary cell survival that operates via GR activation in both nontumorigenic MECs and breast cancer cell lines. The relationship between GR activation and several induced and repressed genes was evaluated by array analysis. One of the GR-induced genes, sgk, encodes a known serum and glucocorticoid-regulated kinase with strong homology in its catalytic domain to the catalytic domain of the antiapoptotic kinase AKT (5). In a panel of MEC cell lines, MCF10A-Myc cells and MDA-MB-231 cells were found to be most sensitive to growth factor withdrawal-induced apoptosis and also demonstrated the lowest endogenous levels of SGK expression (data not shown). Glucocorticoid treatment of these cell lines significantly induced sgk mRNA and resulted in survival. Furthermore, ectopically expressed SGK blocked cell death after growth factor withdrawal, but overexpression of a kinase-dead SGK did not protect cells from apoptosis. The induction of sgk mRNA resulting from glucocorticoid treatment suggests an important transcriptional control mechanism for the activity of this protein. This finding provides a novel model
of kinase activation that appears partially independent of cell surface growth factor receptor signaling. However, the downstream targets of SGK kinase activity remain to be identified.

Interestingly, although SGK activity in human embryonic kidney 293 cells has been shown to be regulated by reversible PI 3-kinase-dependent phosphorylation (6, 12), our results suggest that endogenous PI 3-kinase activity may be sufficient to activate SGK. One possibility is that endogenous phosphoinositide-dependent kinase 1 activity, a downstream target of PI 3-kinase, is particularly high in MECs. Alternatively, a parallel phosphoinositide-dependent kinase 1-like kinase may be responsible for SGK activation in these cells. A third possible explanation is that human MECs may express mutated or low activity phosphatases that would otherwise reverse endogenous PI 3-kinase activity.

The mechanism through which SGK prevents apoptosis remains unknown. However, in kidney epithelium, SGK has been demonstrated to be a target of aldosterone-induced regulation of electrogenic sodium absorption (7, 19, 20), implicating SGK in the control of intracellular fluid volume. Since cell shrinkage is known to be an early hallmark of apoptosis (21), SGK expression may counteract the early changes in cell volume that precede apoptosis by controlling intracellular fluid shifts. The possibility that SGK may exert its antiapoptotic effects by regulating cation channels and maintaining intracellular volume is the subject of future investigation.

In summary, we have identified GR activation as initiating a potent survival signal in both nonmalignant and malignant human breast epithelial cells. SGK, a novel member of the second-messenger family of serine/threonine protein kinases, has been identified as a probable downstream effector of GR survival signaling. Regulation of SGK’s antiapoptotic activity in breast epithelial cells was found to be largely independent of insulin or EGF stimulation, although PI 3-kinase activity is required. Furthermore, transcriptional control of SGK expression appears to be the dominant mechanism of induction of SGK activity, suggesting a novel interaction between steroid hormone receptor activation and serine/threonine kinase-mediated survival.

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REFERENCES
1. Wylie, A. (1980) Nature 284, 555–556
2. Schwartman, R., and Cidlowski, J. (1994) Int. Arch. Allergy Immunol. 105, 347–354
3. Moran, T., Gray, S., Mikosz, C., and Conzen, S. (2000) Cancer Research 60, 867–872
4. Evans-Stroms, R., and Cidlowski, J. (2000) Endocrinology 141, 1854–1862
5. Webster, M., Goya, L., Ge, Y., Maiyar, A., and Firestone, G. (1993) Mol. Cell. Biol. 13, 2031–2040
6. Kobayashi, T., and Cohen, P. (1999) Biochem. J. 339, 319–328
7. Naray-Fejes-Toth, A., Canessa, C., Cleaveland, E., Aldrich, G., and Fejes-Toth, G. (1999) J. Biol. Chem. 274, 16973–16978
8. Alliston, T., Maiyar, A., Buse, P., Firestone, G., and Richards, J. (1997) Mol. Endocrinol. 11, 1934–1949
9. Waldegger, S., Barth, P., Raber, G., and Lang, F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4440–4445
10. Bell, L., Leong, M., Kim, B., Wang, E., Park, J., Hemmings, B., and Firestone, G. (2000) J. Biol. Chem. 275, 25262–25272
11. Kennedy, S., Wagner, A., Conzen, S., Jordan, J., Bellacosa, A., Tsichlis, P., and Hay, N. (1997) Genes Dev. 11, 701–713
12. Park, J., Leong, M., Buse, P., Maiyar, A., Firestone, G., and Hemmings, B. (1999) EMBO J. 18, 3024–3033
13. Horwitz, K., Zava, D., Thilagar, A., Jensen, E., and McGuire, W. (1978) Cancer Res. 38, 2434–2437
14. Wu, K., and Pfahl, M. (1988) Mol. Endocrinol. 2, 1294–1301
15. Nordeen, S., Kuhnel, B., Lawler-Heavner, J., Barber, D., and Edwards, D. (1989) Mol. Endocrinol. 3, 1270–1278
16. Cairns, C., Cairns, W., and Okret, S. (1993) DNA Cell Biol. 12, 695–702
17. Lamontagne, N., Merrier, L., Pons, M., Thompson, E., and Simons, S. J. (1984) Endocrinology 114, 2252–2263
18. Richard-Foy, H., Sistare, F., Riegel, A., Simons, S. J., and Hager, G. (1987) Mol. Endocrinol. 1, 659–665
19. Chen, S., Bhargava, A., Mastroberardino, L., Meijer, O., Wang, J., Buse, P., Firestone, G., Verrey, F., and Pearce, D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2514–2519
20. Shigaev, A., Asher, C., Latter, H., Garty, H., and Reuveny, E. (2000) Am. J. Physiol. Renal. Physiol. 278, 613–619
21. Kerr, J., Wylie, A., and Currie, A. (1972) Br. J. Cancer 26, 239–257
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Christina A. Mikosz, Deanna R. Brickley, Melinda S. Sharkey, Timothy W. Moran and Suzanne D. Conzen

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