The serum- and glucocorticoid-inducible kinase (sgk) is a novel serine/threonine protein kinase that is transcriptionally regulated in rat mammary tumor cells by serum under proliferative conditions or by glucocorticoids that induce a G1 cell cycle arrest. Our results establish that the subcellular distribution of Sgk is under stringent cell cycle and hormonal control. Sgk is localized to the perinuclear or cytoplasmic compartment as a stringently hyperphosphorylated and resided in the nucleus. Laser scanning cytometry, which monitors Sgk localization and DNA content in individual mammary tumor cells of an asynchronously growing population, revealed that Sgk actively shuttles between the nucleus (in S and G2/M) and the cytoplasm (in G1) in synchrony with the cell cycle. In cells synchronously released from the G1/S boundary, Sgk localized to the nucleus during progression through S phase. The forced retention of exogenous Sgk in either the cytoplasmic compartment, using a wild type sgk gene, or the nucleus, using a nuclear localization signal-containing sgk gene (NLS-Sgk), suppressed the growth and DNA synthesis of serum-stimulated cells. Thus, our study implicates the nuclear-cytoplasmic shuttling of sgk as a requirement for cell cycle progression and represents a novel convergence point of anti-proliferative and proliferative signaling in mammary tumor cells.

A dynamic balance of steroid hormones, protein growth factors, and other environmental cues coordinately regulates an intricate network of intracellular processes that stringently control mammalian cell proliferation (1–5). A large body of literature has characterized the individual cellular events activated by either steroid hormones (6–10) or by protein growth factors (3, 11–15), which trigger the two principal signal transduction pathways that eukaryotic cells employ to respond to external stimuli. To understand the functional connections between the transcriptional events regulated by nuclear steroid receptors (7, 8, 10, 16, 17) and the cascades of phosphorylation-dephosphorylation reactions mediated by the cell-surface growth factor receptors (3, 11, 13, 15, 18), a crucial issue was to define the key steps at which these signal transduction pathways converge. There are a variety of potential mechanisms of cross-talk between growth factor and steroid-responsive pathways (5, 19). These regulatory steps include steroid-mediated changes in the expression of growth factors, their cognate receptors, and components of phosphorylation and dephosphorylation cascades (20–29). In other cellular contexts, the phosphorylation of steroid receptors can alter their function and target gene specificity (30, 31). The regulation of cell signaling events in the nucleus for the coordinate control of target genes allows cells to respond to external stimuli in a physiologically appropriate manner. The nuclear import of protein kinases provides one mechanism for modulating cellular signal transduction pathways that functionally complement or potentially couple multiple growth factor pathways to steroid receptor signaling. Several classes of protein kinases, such as protein kinase A, certain isoforms of protein kinase C, mitogen-activated protein kinase, Jun N-terminal kinase, and p90rsk, can translocate to the nucleus and phosphorylate a select group of transcription factors, such as c-Jun and c-Fos components of the AP-1 complex, whose expression and/or activity is targeted by steroid receptor signaling (14, 32–36). Several of the protein kinases implicated in the nuclear cross-talk between steroid receptor signaling and growth factor-induced pathways are involved in the regulation of proliferative or anti-proliferative responses. For example, in T cells, glucocorticoids down-regulate the phosphorylation of the basal and activated forms of the p70s6k/p85s6k kinase (37), a protein kinase whose activity and phosphorylation is regulated during cell cycle progression (38). However, relatively little is known about the functional coordination between the expression and localization of protein kinases involved in cell signaling and the hormonal control of cell proliferation.

Glucocorticoids have been shown to inhibit the growth and regulate cell cycle progression of many different cell types (5, 9), with their most characterized effects shown in mammary tumor cells (39–41), hepatoma cells (42, 43), osteosarcoma cells (44), and lymphoma cells (45). To investigate directly mechanistic relationships between nuclear growth factor and steroid

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cell derived tumors (47), induce the formation of tight junctions actively progressing through the cell cycle. We have documented that glucocorticoid hormones strongly suppress Con8.hd6 cell growth by inducing a specific G1 block in cell cycle progression (41), inhibit the in vivo growth of Con8-cell derived tumors (47), induce the formation of tight junctions (48, 49), and inhibit the production of an autocrine transforming growth factor-α (21) which acts through epidermal growth factor receptor-mediated phosphorylation cascades (50). Subtractive cloning of glucocorticoid-responsive genes from a Con8 mammary tumor cell cDNA library was used to identify genes involved in the glucocorticoid-mediated growth suppression response. We isolated a novel serum and glucocorticoid-inducible serine/threonine protein kinase gene, sgk, which is transcriptionally regulated by glucocorticoids and serum (22, 23). The sgk promoter contains a functional glucocorticoid response element that accounts for its glucocorticoid inducibility (51) and is a transcriptional target of the p53 tumor suppressor protein (52). The existence of this novel transcriptionally regulated protein kinase suggests a new pathway of cross-talk by which glucocorticoid receptor-mediated and phosphorylation-dependent cell signaling can be coordinated and therefore implicates Sgk as a potential target in the control of cell proliferation.

The sgk gene encodes a 50-kDa protein with 431 amino acids that shows strong homology (45–54% amino acid identity) to the catalytic domains of other serine/threonine protein kinases, such as Akt (54%), the rat p70S6k/p85S6K kinases (50%), rat protein kinase C-β (48%), and the mouse protein kinase A (45%) (22). Several members of these protein kinase gene families that are homologous to sgk are involved in cell signaling events that are associated with the control of cell growth and differentiation (3, 5, 15, 34, 53). The phosphorylation, activity, and in some cases the cellular localization of these protein kinases can be regulated in response to specific extracellular stimuli. Thus, even though sgk gene expression is under an acute transcriptional control by several distinct pathways, the cellular utilization of Sgk protein may also be highly regulated under conditions in which the mammary tumor cells are either growth-arrested by glucocorticoids or actively proliferating in the presence of serum. In this study, we demonstrate that the nuclear versus cytoplasmic localization of Sgk is under stringent hormonal and cell cycle control, and we also provide evidence that the nuclear-cytoplasmic shuttling of Sgk may be required for the ability of the mammary tumor cells to be actively progressing through the cell cycle.

**EXPERIMENTAL PROCEDURES**

**Cell Culture Conditions and Radiolabeling—**Con8.hd6 mammary epithelial tumor cells were routinely cultured on standard tissue culture plates in DMEM/F-12 (BioWhittaker) supplemented with 10% fetal bovine serum (BioWhittaker) and maintained at 37 °C and 5% CO₂ as described (39, 40). The cells were incubated with serum-free medium prior to their treatment with the indicated concentrations of serum and/or 1 μM dexamethasone for the indicated times. In the appropriate experiments, 5-bromo-2'-deoxyuridine (BrdUrd) (Sigma) was added to a final concentration 100 μM for 30 min. For cell synchronization, hydroxyurea was added to the final concentration of 1 mM to 30% confluent cells for 24 h, after which 95% of the cell population was found to be in G1 phase of the cell cycle, as determined by flow cytometry. For radiolabeling with 32P[orthophosphate (10 μCi/ml H₂O, NEN Life Science Products), Con8.hd6 cells were grown to 30% confluency before they were serum-deprived for 48 h. The cells were then incubated in phosphate-free DMEM for 1 h, followed by 1 μCi/ml fucose in phosphate-free DMEM supplemented with the indicated combinations of serum-free conditions, 10% dialyzed calf serum, and/or 1 μM dexamethasone for 4 h. Cells were pulse-labeled for 1 h with 0.5 mCi of carrier-free 32P[orthophosphate in 4 ml of incubation medium. Cells were harvested, and Sgk was immunoprecipitated using the affinity purified polyclonal anti-Sgk antibodies and protein A beads (Pierce). Immunoprecipitated Sgk beads were resolved by SDS-PAGE, followed by autoradiography.

**Generation of Polyclonal Anti-Sgk Antibodies and Affinity Purification—**To express Sgk in bacteria, the pET-HAX-Sgk expression vector was generated for isopropyl-1-thio-β-d-galactopyranoside-induced Sgk expression in HMS174. For this purpose, an NdeI fragment containing the full-length and sequenced sgk open reading frame was inserted into pET-HAX vector. The vector harbors the sequence that encodes a hemagglutinin (HA) tag. The sgk cDNA beginning with the initial Met was inserted into the NdeI site, and its N terminus was thus fused in frame to the HA tag. Milligram quantities of HA-tagged Sgk were expressed in HMS174 bacteria that had been transformed with the pET-HAX-Sgk expression construct. After induction with isopropyl-1-thio-β-d-galactopyranoside, cells were harvested, and lysed in HEMGN buffer (25 mM Hepes, pH 7.4, 150 mM NaCl, 0.05% Tween 20, 100 mM KCl, 12.5 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, pH 7.9) homogenization buffer; the lysate was size-fractionated by SDS-PAGE, and the major Sgk band at 50 kDa was excised. The SDS-PAGE gel slice was then quick-frozen and homogenized. The gel particles were mixed with Freund’s adjuvant, filtered, and inoculated into female New Zealand White rabbits. Serum was extracted from the immunized rabbits at standard intervals to monitor antibody titer (Babcro, Richmond, CA). The final titer of the unfractionated serum was high enough to detect sgk at a 1:10,000 dilution on a Western blot of whole cell lysate from glucocorticoid-treated Con8-hd6 cells not overexpressing Sgk. For the purification of anti-Sgk antibodies by affinity column chromatography, bacterially expressed His-tagged Sgk was coupled to a nickel column (Ni²⁺-nitrilotriacetic acid-agarose) (Qiagen, Chatsworth, CA). The anti-Sgk serum was repeatedly applied to the column, and the specifically bound anti-Sgk antibodies were eluted with 50 mM glycine, 0.1 M NaCl, pH 2.3 into 1 M Tris, pH 9.0, for neutralization and dialyzed against HEMGN. The purified antibodies were tested by Western blot analysis and shown to recognize multiple forms of sgk that migrated between 50 and 52 kDa.

**Western Blot Analysis—**Whole cell extracts (20–50 μg of protein) were electrophoretically resolved by SDS-PAGE, and the proteins were transferred to Nitran Plus membranes (Schleicher & Schuell). The membrane was probed with a 1:5,000 dilution of polyclonal anti-Sgk antibody in 5% nonfat dry milk. The secondary antibody was a goat anti-rabbit IgG horseradish peroxidase-conjugated antibody (Bio-Rad) and was visualized with 1:1,000 Western blotting developer using the Renaissance developing kit (NEN Life Science Products) and exposed to x-ray film.

**Phosphatase Treatment of Immunoprecipitated Sgk—**Cells ectopically expressing His-tagged Sgk were harvested in HEMGN buffer containing 10 mM imidazole and the lysate incubated with Ni²⁺-nitrilotriacetic acid-agarose. The manufacturer’s recommendations were followed to affinity purify His-tagged Sgk. The beads were equilibrated in 40 mM PIPES, pH 6.0, 1 mM dithiothreitol, 20 μg/ml aprotinin, 20 μg/ml leupeptin and incubated in the presence and absence of 100 μM potato acid phosphatase (Sigma) for 30 min at 30 °C. Final beads were washed once and resolved by SDS-PAGE, followed by Western blotting with polyclonal anti-Sgk antibodies.

**Indirect Immunofluorescence Microscopy for Sgk Localization and Incorporation of Bromodeoxyuridine—**Con8.hd6 cells were cultured on 8-well Lab-Tek Permanox slides (Nalgene Nunc International, Naperville, IL) or on sterile cover slides and grown to 30% confluency before the indicated combinations of serum and/or dexamethasone were added for the indicated time frame. Cell confluency prior to fixation did not exceed 80%. For indirect immunofluorescence microscopy, cells were fixed with PB, fixed for 15 min in 3.7% formaldehyde, 0.1% glutaraldehyde, rinsed with PBS, and permeabilized with 50% methanol, 50% acetone for 1 min. Following a rinse in PBS, the cells were preabsorbed for 5 min in PBS containing 4% normal goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Cells were incubated with a 1:300 dilution of affinity purified rabbit polyclonal anti-Sgk antibody for 1–2 h at 25 °C. After 5 washes with PBS, the cells were
treated for 5 min with PBS containing 4% normal goat serum. The cells were incubated with a 1:300 dilution of anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (Cappel Research Products, Durham, NC) in PBS and then incubated for 30 min at 25 °C. Cells were washed 5 times with PBS and mounted with 50% glycerol, 50 mM Tris, pH 8.0, containing 0.1 mg/ml propidium iodide and 0.1% Triton X-100. Fluorescence was monitored by indirect immunofluorescence microscopy using a Nikon Optiphoto fluorescence microscope. Images were captured using Adobe Photoshop 3.0.5 (Adobe Systems, Inc., Mountain View, CA) and a Sony DKC-5000 digital camera. Non-specific fluorescence was determined by incubation with the secondary antibody alone and shown to be negligible. Due to the use of an affinity purified antibody, preimmune serum showed a relatively higher background and was not used as a negative control.

Double labeling for Sgk localization and the incorporation of 5-bromo-2'-deoxyuridine (BrdUrd) utilized the above procedures with the following modifications. During the final hour of incubation in medium containing the indicated combinations of serum and/or dexamethasone, the cells were incubated in medium containing a final concentration of 100 μM BrdUrd (Sigma) at 37 °C for 30 min. Following fixation, the cells were rinsed with PBS, and the DNA was denatured by incubation in 0.12 N HCl at 37 °C for 1 h. After neutralization in two changes of 0.1 M borate buffer over 10 min, cells were washed 3 times for 30 min and then incubated in PBS containing 4% normal goat serum. The addition of 20 μg/ml of monoclonal anti-BrdUrd antibody (Dako Corp., Carpinteria, CA) in PBS for 60 min at 25 °C. After the washes, secondary antibodies were added as described above with the addition of a 1:300 dilution of anti-mouse rhodamine-conjugated secondary antibody (Jackson Immunoresearch) in PBS and incubated at 25 °C for 30 min.

Flow Cytometry—Con8-hd6 cells were cultured in 6-cm plates, not exceeding 35% confluency. The cells were washed twice with PBS and suspended in 500 μl of propidium iodide solution (75 μM propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100). Finally, cells were filtered through a polycrylamide mesh and maintained on ice until use. Nuclear-emitted fluorescence was measured with a Coulter Elite instrument with laser output adjusted to deliver 15 milliwatts at 488 nm. Cell nuclei (10⁶) were analyzed from each sample at a rate of 300–500 nuclei/s. The percentages of cells within the G₁, S, and G₂/M phases of the cell cycle were determined by analysis with the Multicycle computer program provided by Phoenix Flow Systems in the Cancer Research Laboratory Microchemical Facility of the University of California, Berkeley. The data were collected and analyzed with a Becton Dickinson FACScan and Lysis II software (Becton Dickinson).

Laser Scanning Cytometry—The cells were analyzed on a laser scanning cytometer (CompuCyte Corp., Cambridge MA) using argon ion laser excitation at 488 nm. Fluorescence was collected by photomultiplier tubes with green (530 DF 30) and red (600 DF 60) band pass filters, digitized to 0.25 μm by 0.5-μm pixel resolution, and temporarily stored as computer memory images. When complete memory images were obtained, they were segmented into nuclei using image processing techniques. The pixel values were calculated for each cell, including the total DNA content, and amount of green fluorescence, and stored in a list mode computer data file. Green fluorescence was calculated twice, once for the region containing the cell nucleus and second for a torus extending out from the nucleus to sample the cytoplasm of the cell. By plotting these features in two parameter dot plots, it was possible to define regions around clusters of cells with nuclear fluorescence, and regions around cells with cytoplasmic fluorescence, and color gate cells from these regions onto one-parameter DNA histograms. Cells from the specific regions were also relocated and visualized by epifluorescence illumination. The scanning is done in an automated fashion, with 5,000–10,000 cells analyzed per sample.

Generation of Wild Type and Nuclear Localization Signal-containing sgk Expression Vectors—The pcDNA3 mammalian expression plasmid (Invitrogen, Carlsbad, CA) with the full-length sgk cDNA was utilized for the ectopic expression of Sgk in cell culture. Standard PCR techniques were used starting with the full-length wild type rat sgk cDNA (22). The final expression vector contains an Asp-718 site and the first codons of the sgk sequence, and 5'-TTCTGATTAATGTATGTATGTATGTATGGAGGAAGGG-AGTCCATAGCAGG-3', which contains the XhoI site, the Stop-codon, eight His-residues, and the final amino acids of the Sgk C terminus.
Clear or cytoplasmic fluorescent pattern, as observed by a brightly staining ring around the nuclear membrane. Similar to cells treated only with glucocorticoids, mammary tumor cells treated with dexamethasone and serum harbor Sgk exclusively in a perinuclear/cytoplasmic location (Fig. 1, lower right panel). Cells maintained in a serum-deprived state have a low level of diffuse staining of Sgk (Fig. 1, top left panel) that is only slightly more intense than that observed when fixed cells are incubated with secondary antibodies and primary anti-Sgk antibody (data not shown). The faint homogeneous staining observed in serum-deprived cells may be due to a low level of expressed Sgk since significant overexposure of Western blots revealed a small amount of the 50-kDa Sgk protein.

The differential effects of serum and dexamethasone on Sgk localization was quantitated by visually assessing the subcellular localization of Sgk in at least 500 cells per condition. In order to visually quantify individual cells, the mammary tumor cells were cultured at very low confluency (10–20%) in the absence of serum and dexamethasone for an 8-h time course. Western blots revealed a small amount of the 50-kDa Sgk protein. The exclusive localization of Sgk in the perinuclear-cytoplasmic compartment of cells treated for 48 h with both serum and dexamethasone was approximately equal to that observed in cells treated only with dexamethasone for 18 h in the absence of serum. Cells that were incubated only with serum only displayed perinuclear-cytoplasmic staining of Sgk (Fig. 2, middle set of bar graphs). The opposing effects of serum and glucocorticoid on Sgk localization were completely reversible. For example, incubation of serum-treated cells with dexamethasone caused Sgk to redistribute from a predominantly nuclear localization to a perinuclear-cytoplasmic location, whereas additional treatment of these cells with a potent glucocorticoid antagonist, RU486, in the presence of serum resulted in the movement of Sgk back to the nuclear compartment (data not shown).

Sgk is Phosphorylated after Serum Stimulation but Not after Glucocorticoid Treatment—Because sgk is differentially localized in serum-stimulated cells compared with glucocorticoid-treated cells, the potential modification of Sgk was examined in mammary tumor cells stimulated with high (10%) or low (0.5%) serum. The peak expression of Sgk in low serum rapidly stimulated the expression of a 50-kDa Sgk species that was continuously present in cells during the 8-h time course (Fig. 3, lower left of panel A) and was maintained at high levels even after 48 h steroid treatment (data not shown). In contrast, stimulation of serum-deprived cells with 10% serum is accompanied by the rapid and transient induction of differentially migrating forms of Sgk, including the 50-kDa species as well as larger forms (up to 52 kDa) of Sgk (Fig. 3, top right of A).

Treatment of serum-deprived cells with a combination of serum and dexamethasone for 18 h resulted in a heterogeneous staining pattern in which 25% of cells harbored Sgk in the nucleus, 15% were found to have Sgk in their nuclei as well as in the cytoplasm (homogeneous staining), and 65% of cells displayed exclusively perinuclear-cytoplasmic staining of Sgk (Fig. 2, middle set of bar graphs). A 48-h treatment with both serum and dexamethasone resulted in virtually all cells producing Sgk as a perinuclear-cytoplasmic staining protein that coincided with the loss of the hyperphosphorylated form of Sgk. Because the exclusive localization of Sgk in the perinuclear-cytoplasmic compartment of cells treated for 48 h with both serum and dexamethasone was approximately equal to that observed in cells treated only with dexamethasone for 18 h in the absence of serum, Sgk was quantitated and expressed as the percentage of total immunostaining cells. The error bars indicate the standard deviations.
treated cells varied by their degree of phosphorylation and did not represent distinct isofoms, poly-His-tagged Sgk was transiently overexpressed in the mammary tumor cells, affinity purified by binding to a nickel matrix, and incubated in the presence or absence of potato acid phosphatase. Western blot analysis of Sgk showed that potato acid phosphatase digestion caused the multiple forms of Sgk to collapse into a single protein species with a molecular mass of approximately 50 kDa (Fig. 3B, blot). This apparent loss of Sgk immunoreactive protein following phosphatase treatment is likely due to a change in antigenicity resulting from its complete dephosphorylation in vitro. Furthermore, we have recently observed that incubation with either wortmannin or LY294002 inhibited the production of the slower migrating forms of Sgk, which suggests that the regulation of Sgk phosphorylation is a downstream target of the cytoplasmic phosphatidylinositol 3-kinase pathway.\textsuperscript{2} Taken together, our results show that the distinct electrophoretic migration of Sgk produced in serum-treated mammary tumor cells resulted from differential phosphorylation. Moreover, the formation of the hyperphosphorylated forms of Sgk correlated with the localization of sgk into the nucleus under serum-stimulated conditions, whereas a hypophosphorylated form of sgk was detected under conditions in which sgk was localized to the cytoplasm in glucocorticoid treated cells.

**Nuclear Localization of Sgk in S Phase Mammary Tumor Cells**—The differential subcellular distribution and phosphorylation of Sgk in serum-stimulated versus glucocorticoid growth-arrested mammary tumor cells suggested that the regulation of Sgk localization may be closely linked to the steroid and growth factor control of the cell cycle. As an initial test whether the nuclear localization of Sgk during serum stimulation coincides with entry into S phase, cells were labeled with the thymidine nucleotide analog BrdUrd, and cells were stained for both Sgk and for the incorporation of BrdUrd. Double staining of individual cells was achieved by using fluorescein-labeled secondary antibodies that recognize the anti-Sgk primary antibodies and rhodamine-conjugated secondary antibodies that selectively bind to the anti-BrdUrd primary antibodies. As shown in Fig. 4 (left column of panels), essentially all of the 18-h serum-stimulated mammary tumor cells that are actively synthesizing DNA harbor Sgk in the nucleus as shown by the striking co-staining of Sgk and BrdUrd. A fraction of the cells did not incorporate BrdUrd but still localized Sgk to the nucleus and, as shown in a later section, represent cells in G\textsubscript{2}/M. In contrast, cells that were growth-suppressed after treatment with both serum and dexamethasone for 24 h failed to incorporate BrdUrd and selectively expressed Sgk as a perinuclear-cytoplasmic protein (Fig. 4, right column of panels).

The subcellular distribution of Sgk was quantitated in the S phase cells within a large population of mammary tumor cells. The mammary tumor cells were initially serum-deprived for 48 h. BrdUrd-positive cells were then scored after treatment with only serum for 18 h, with serum and dexamethasone for 18 h, and with both extracellular signals for 48 h. At least 500 cells per condition were examined, and the relative numbers of BrdUrd-positive cells were found to match closely the relative number of cells with an S phase DNA content (48) and the level of [\textsuperscript{3}H]thymidine incorporation (41). An 18-h serum stimulation, 410 cells stained positive for BrdUrd incorporation. Of those cells, 50% exhibited nuclear staining of Sgk, 38% of cells were homogeneously stained, and 12% displayed periplasmic or cytoplasmic staining (Fig. 5, left bar graphs). The homogeneously stained cells expressed Sgk to approximately the same

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\textsuperscript{2} P. Buse and G. L. Firestone, unpublished results.
extent in the nucleus and in the cytoplasm. Combining the nuclear and homogeneous categories revealed that 88% of BrdUrd-positive cells harbor Sgk in the nucleus. Treatment with dexamethasone and serum for 18 h reduced by approximately one-half the number of BrdUrd-positive cells, which reflects the partially growth-arrested state. In this smaller number of S phase cells, the same relative proportion of nuclear-localized Sgk was maintained as that observed with the serum-stimulated cells. Ninety percent of S phase cells expressed Sgk in the nucleus with 40% of BrdUrd-positive cells displaying exclusively nuclear Sgk and 50% showing homogeneous staining (Fig. 5, middle bar graphs). Taken together, our results indicate that Sgk localization may be regulated in a cell cycle-dependent manner because this protein kinase is distributed to the nucleus in cells actively synthesizing DNA and into a perinuclear/cytoplasmic compartment in G1 growth-arrested cells.

Determination of Sgk Subcellular Localization and Cell Cycle Phase in an Asynchronous Cell Population by Laser Scanning Cytometry—Conceivably, the nuclear localization of Sgk observed in serum-stimulated cells and its perinuclear/cytoplasmic localization in glucocorticoid-treated cells arrested in G1 results from the cell cycle phase-regulated compartmentalization of this protein kinase. To test directly this possibility, laser scanning cytometry was utilized to simultaneously determine the subcellular distribution of Sgk and the DNA content of a large number of individual cells in an asynchronously growing population. Serum-stimulated cells were fixed and then simultaneously stained for Sgk and for DNA content by propidium iodide. Five thousand to ten thousand mammary tumor cells were individually examined for Sgk localization in the nuclear (green profile) or perinuclear (red profile) compartments and for the corresponding DNA content (black profile) by laser scanning cytometry. As shown in Fig. 6, the overall distribution of cellular DNA content (shown in black) is of an asynchronous growing population of cells. The nuclear-associated Sgk (green) strongly correlates with cells containing a DNA content indicative of S and G2/M phase cells. Interestingly, Sgk was primarily localized to the nucleus in S phase cells, whereas cells in G2/M displayed a more diffuse nuclear staining pattern. The nuclear localization of Sgk in this population of mitotic cells explains the observation that Sgk can be nuclear-associated in a subset of cells that do not incorporate BrdUrd (see Figs. 4 and 5). As also shown in Fig. 6, in contrast, the cells with a G1 DNA content in the growing population of serum-treated cells expressed Sgk in the cytoplasmic compartment (red) suggesting that the phase of the cell cycle regulates Sgk localization. Consistent with our previous flow cytometry results (41, 48), dexamethasone growth-arrested cells accumulated with a predominantly G1 DNA content and expressed Sgk.
cells after release from a G1/S block in cell cycle progression. The distribution was examined in serum-treated mammary tumor cells regulated by progression through S phase of the cell cycle, Sgk.

To verify that the nuclear localization of Sgk can be achieved, the 24-h incubation in hydroxyurea resulted in 64% of cells entering G2/M phase. At each time of release, cells were co-stained for Sgk localization and DNA content using a laser scanning cytometer with an argon ion laser excitation at 488 nm. On an individual cell basis, the localization of Sgk was determined and compared with the corresponding cellular DNA content. The quantitation of cellular DNA content within the cell population is shown in the black profile. The number of cells with cytoplasmically localized Sgk is shown in red, and the nuclear localized Sgk is shown in green. The lower panel represents a magnified portion of the upper panel.

DNA Content

**Fig. 6.** Laser scanning flow cytometric analysis of Sgk localization in an asynchronously growing population of mammary tumor cells. A growing population of serum-treated cells was fixed and stained for Sgk localization using affinity purified rabbit polyclonal primary antibodies to Sgk. During the incubation with the fluorescein isothiocyanate-conjugated secondary antibodies, the fixed cells were also treated with propidium iodide to stain the nuclear DNA. Individual cells within the cell population (10,000 cells per sample) were simultaneously examined for Sgk localization and DNA content using a laser scanning cytometer with an argon ion laser excitation at 488 nm. On an individual cell basis, the localization of Sgk was determined and compared with the corresponding cellular DNA content. The quantitation of cellular DNA content within the cell population is shown in the black profile. The number of cells with cytoplasmically localized Sgk is shown in red, and the nuclear localized Sgk is shown in green. The lower panel represents a magnified portion of the upper panel.

as a perinuclear-cytoplasmic protein (data not shown). Based on these results, we propose that Sgk localization is regulated by the phase of the cell cycle as well as by the cell cycle effects of growth factor and glucocorticoid treatment. Sgk is observed to be perinuclear-cytoplasmic during progression through the G1 phase, whereas during the onset of S phase and progression into G2/M of the cell cycle, Sgk is mostly nuclear.

**Sgk Localizes to the Nucleus during Progression through S Phase**—To verify that the nuclear localization of Sgk can be regulated by progression through S phase of the cell cycle, Sgk distribution was examined in serum-treated mammary tumor cells after release from a G1/S block in cell cycle progression. The cells were first blocked at the G1/S boundary by a 24-h incubation with hydroxyurea and then allowed to progress through S phase by replacing the hydroxyurea-containing medium with fresh serum-containing medium without hydroxyurea. Flow cytometry of propidium iodide-stained nuclei showed that the 24-h incubation in hydroxyurea resulted in 64% of cells displaying a G1 DNA content, and 35% of cells had an S phase DNA content with minimal cell death (Fig. 7, 0 h release). Replacing the hydroxyurea-containing media with fresh serum-containing media without hydroxyurea allowed the cell population to synchronously progress through the cell cycle. Four hours after release from hydroxyurea treatment, approximately 90% of the cell population was in S phase, and by 8 h release a large fraction of the cell population had progressed into G2/M phase. At each time of release, cells were co-stained by immunofluorescence for Sgk localization and for the incorporation of BrdUrd. As shown in Fig. 7, at the G1/S boundary (0 h release time) Sgk displayed a diffuse cytoplasmic staining pattern with negligible incorporation of BrdUrd. After 4 h of serum stimulation and subsequent release from hydroxyurea treatment, Sgk was localized predominantly to the nuclear compartment under conditions in which virtually all of the cells had entered S phase and incorporated BrdUrd. Eight hours after release, significantly fewer cells incorporated BrdUrd and the sgk-staining pattern was still mostly nuclear with a more diffuse staining pattern. In a control set of plates, in which the cells were also exposed to fresh serum-containing medium, but with continued incubation with hydroxyurea, the cells failed to enter S phase and Sgk remained as a weakly staining, cytoplasmic protein (data not shown). Thus, progression through S phase, rather than the growth factor signaling stimulated by exposure to fresh serum, caused Sgk to become localized to the nucleus which likely accounts for the observed serum regulation of Sgk distribution and formation of hyperphosphorylated forms of Sgk.

**The Anti-proliferative Effects of Sgk Ectopically Expressed in the Nuclear or Cytoplasmic Compartments**—To determine if the targeted Sgk localization affects the proliferative state, mammary tumor cells were transfected with either a wild type sgk cDNA expression vector or with an sgk expression vector in which the SV40 large T antigen nuclear localization signal is...
fused to the C terminus of Sgk (forming NLS-Sgk). The presence of the nuclear translocation signal should force Sgk to be nuclear-associated in transfected cells. Each form of the sgk gene was His-tagged at the C terminus and driven by the CMV promoter. Western blots confirmed that the exogenously expressed Sgk proteins were full-length and modified similarly to the endogenous protein, including the presence of the more slowly migrating phosphorylated forms of Sgk after serum treatment (data not shown). The subcellular distribution of Sgk and BrdUrd incorporation were monitored by co-immunofluorescence in a serum-treated population of transiently transfected cells. Since commercially available anti-His antibodies failed to detect the His-tagged Sgk, immunocytochemistry was performed using a 1:800 dilution of the affinity purified polyclonal antibodies to Sgk which is a concentration below the threshold level required to detect endogenously expressed Sgk but is sufficient to detect the high levels of overexpressed Sgk protein. As shown in the upper panels of Fig. 8, exogenously expressed wild type Sgk (WT-sgk) was exclusively localized to the cytoplasmic compartment and did not harbor Sgk in the nucleus. However, the NLS-Sgks was localized primarily to the nuclear compartment with virtually no staining observed in the cytoplasm. Approximately 5% of the subconfluent monolayer of cells produced the ectopically expressed forms of Sgk (Fig. 8, upper panels). For either the WT-Sgk or NLS-Sgk constructs, the subset of cells that ectopically express Sgk generally failed to incorporate BrdUrd (Fig. 8, upper versus lower panels). Conversely, the cells that stain positive for BrdUrd do not produce either the WT-Sgk or the NLS-Sgk. A small subset of the NLS-Sgk-expressing cells were shown to incorporate a low level of BrdUrd; however, in general, the production of nuclear-associated Sgk did not drive the overexpressing cells into S phase. Based on the laser scanning cytometric analysis of endogenous Sgk, in which this kinase is nuclear-associated in either the S or G2 phases of the cell cycle, it is possible that the cells that ectopically express NLS-Sgk may reside in G2 phase and therefore do not incorporate BrdUrd. These results show that cells ectopically expressing either a cytoplasmic-residing Sgk or a nuclear-residing Sgk are not observed to enter S phase and that the nuclear association of Sgk is not sufficient to induce proliferation of the mammary tumor cells.

A colony formation assay was employed to determine if ectopic expression of the WT-Sgk or NLS-Sgk can suppress the growth of a population of transfected mammary tumor cells. In this assay, cells were co-transfected with either WT-Sgk or NLS-Sgk expression vectors or with an empty vector (vector control) along with the neomycin resistance gene, and identical numbers of transfected cells were plated in selective medium. After a week, the cell cultures were stained to visualize the surviving cell colonies (Fig. 9). The combined results from five independent experiments were quantitated. Ectopic expression of either the WT-Sgk or NLS-Sgk reduced the formation of cell foci by approximately 60% compared with cells that received only the empty vector (Fig. 9). The final recovered cell colonies do not express exogenous Sgk, and consistent with the growth-suppressing effects of this kinase, we have not been able to recover cells that stably overexpress Sgk in these mammary tumor cells (data not shown). Our results from both the BrdUrd labeling of individual cells and from the cell foci population assay show that Sgk can function in an anti-proliferative pathway when ectopically expressed and retained in either the cytoplasmic or nuclear compartments. Since Sgk is observed in the nucleus in S and G2/M phase cells and in the cytoplasmic compartment in G1 phase cells, our results further imply that the nuclear-cytoplasmic shuttling of Sgk may be required for the cell to progress through the cell cycle.

**DISCUSSION**

The growth of normal and transformed cells is controlled by an intricate network of intracellular processes that converge at
Sgk Localization and Regulation of Cell Proliferation

Serum stimulates cell cycle progression (22, 23), whereas glucocorticoids cause a G1 cell cycle arrest (41, 48). The stimulation of sgk gene expression by each extracellular signal implicates this protein kinase as a functioning component of either the serum-stimulated proliferative pathway or of the anti-proliferative response to glucocorticoids. Consistent with this notion, our results have now established that nuclear-cytoplasmic compartmentalization of sgk is differentially regulated depending on the proliferative state of the cells in that sgk is localized to the cytoplasmic compartment in mammary tumor cell growth-arrested by glucocorticoids and is predominantly localized in the nucleus after serum stimulation.

In an asynchronous growing population of mammary tumor cells, Sgk distributed to either the nucleus or the cytoplasmic compartment in synchrony with the phase of the cell cycle. Laser scanning cytometry, which simultaneously monitors DNA content and Sgk localization in individual cells, revealed that Sgk resides in the cytoplasm during the G1 phase of the cell cycle and then is compartmentalized to the nucleus in the S and G2/M phases. This result indicates that the cytoplasmic localization of Sgk in glucocorticoid-treated cells may be due to the specific stage of the cell cycle in which these cells are arrested, rather than by the direct actions of this steroid on sgk localization. The precise location of Sgk within the cytoplasm has not been determined, although based on the subcellular distribution of homologous kinases (33, 34, 53–56), Sgk may either be anchored to cytoplasmic membranes or free in the cytoplasm. Immunofluorescence co-staining for Sgk localization and incorporation of BrdUrd in serum-stimulated cells, or in cells synchronously released from a G1/S block, revealed that virtually all of the cells in S phase express sgk as a nuclear-associated protein. We propose that during cell cycle progression, Sgk shuttles between the nuclear and cytoplasmic compartments at the beginning of G1 and at the G1/S boundary. It is tempting to consider that the shuttling between the nucleus and cytoplasm is required for sgk function in cellular proliferative pathways, whereas its regulated retention in the cytoplasm in growth-arrested cells, for example after the G1 cell cycle arrest mediated by glucocorticoids, allows sgk to function within an anti-proliferative pathway or be sequestered away from a crucial proliferative cascade. Thus, the regulation of Sgk compartmentalization represents a previously uncharacterized convergence point for proliferative or anti-proliferative signaling cascades.

A key prediction for the requirement of nuclear-cytoplasmic shuttling of Sgk in proliferating cells is that the forced retention of this kinase in either the nucleus or the cytoplasm should suppress cell growth. Ectopic expression of either WT-Sgk, the wild type kinase that stably resides in the cytoplasmic compartment, or NLS-Sgk, which stably resides in the nucleus as a result of its nuclear localization signal, suppressed mammary tumor cell growth in a cell foci population assay. Also, immunofluorescence analysis of individual cells producing either WT-Sgk or NLS-Sgk revealed that the cells do not enter S phase. These results showed that the forced retention of Sgk in either the nuclear or cytoplasmic compartments is sufficient to suppress cell growth and that the shuttling of this kinase between these two cell compartments is likely to be important for the mammary tumor cells to proliferate. Conceivably, the regulated translocation of Sgk between the nuclear and cytoplasmic compartments controls the availability of stimulus-specific or cell cycle-regulated signaling components that may modulate the activity of Sgk and potential access to specific substrates. For example, nuclear Sgk could interact with and potentially phosphorylate specific sets of transcription factors that regulate the expression of genes that control proliferative functions, or phosphorylate essential cell cycle factors required during S and G2. The cytoplasmic compartmentalization of Sgk during G1 may exclude Sgk from having access to specific nuclear components during this phase of the cell cycle or may recruit this kinase to a new set of targets in the cytoplasm that help the cells progress through G1. One explanation for the growth suppression induced after ectopic expression of Sgk into either the nucleus or the cytoplasm is that high levels of exogenous Sgk may competitively sequester key components into an inactive complex, which are needed for cell cycle progression, or prevent the active forms from localizing to their site of function.

In other cell systems, the ectopic expression of certain Ser/Thr protein kinases has been shown to attenuate cellular proliferation and/or decrease the tumor formation (57–60) but does not induce a stringent growth arrest.

Our studies show for the first time that the post-translational nuclear-cytoplasmic shuttling of a transcriptionally regulated protein kinase can be regulated at specific stages of the cell cycle. There are only a few precedents for the nuclear localization of Ser/Thr protein kinases that are activated as part of growth factor receptor signaling. Protein kinase C, mitogen-activated protein kinase, p90S6K, and AkT/PI3K have been shown to translocate into the nucleus in order to phosphorylate and activate a number of transcription factors (34, 55, 61). The p70S6K/p85S6K protein kinases, which are 50% identical to Sgk in their catalytic domains, have been shown to be phosphorylated and localized in a cell cycle-dependent manner (38, 53). In S phase in their respective systems, Sgk and p70S6K/p85S6K are predominantly nuclear with a speckled staining pattern indicative of co-localization with heterochromatin (38), whereas both kinases are enriched in the cytoplasm in the G1 phase. Microinjection of rat embryo fibroblasts with antibodies that inhibit p70S6K/p85S6K activity abolished the serum-induced entry into S phase (53), whereas we have shown that ectopic expression of Sgk inhibited cell growth. Similar to Sgk, the phosphorylation of p70S6K/p85S6K has been shown to be reduced after glucocorticoid treatment of T cells (37). The C terminus of Sgk contains a Ser/Thr-Pro recognition motif for cell cycle-regulated kinases, and the same motif with similar spacing between polar amino acid residues is the site of cell cycle-dependent phosphorylation in the homologous p70S6K/p85S6K kinase. Thus, Sgk and p70S6K/p85S6K may be associated with functionally analogous phosphorylation-dephosphorylation pathways that direct progression through the cell cycle.

In serum-stimulated cells, the formation of a hyperphosphorylated form of sgk generally correlated with its localization to the nuclear compartment, whereas at steady state in glucocorticoid-treated cells, sgk was hypophosphorylated and distributed to the cytoplasmic compartment. Phosphorylation or dephosphorylation has been shown to affect directly or to correlate with the nuclear import or export of a wide variety of proteins, including several protein kinases and cell cycle-regulated factors (32, 56, 62–66). Thus, one potential function for the phosphorylation of Sgk in serum-stimulated cells may be to facilitate or alter its nuclear transport at particular phases of the cell cycle or in a stimulus-dependent manner. However, the
phosphorylation of sgk per se does not appear to regulate directly its subcellular distribution because the exogenously introduced wild type sgk, which remains cytoplasmic, and the NLS-sgk, which is retained in the nucleus, are both hyperphosphorylated (data not shown). Also, cellular stress, such as osmotic shock, induces a hyperphosphorylated form of sgk that resides in the cytoplasm. In this regard, it is likely that the phosphorylation of sgk occurs in the cytoplasm because our recent preliminary studies have shown that the phosphorylation of Sgk is regulated by the phosphatidylinositol 3-kinase pathway, and based on the homology of Sgk to Akt (22, 23), it is likely to be a direct target of the 3-phosphoinositide-dependent protein kinase family of cytoplasmic kinases.

The precise mechanism by which sgk is transported between the nucleus and the cytoplasm in a stimulus- or cell cycle-regulated manner is not known, although the cellular control of this shuttling process is likely to be functionally linked to the growth factor, steroid, and cellular stress signal transduction pathways that target sgk. Many proteins that are imported into the nucleus have an identifiable NLS defined by a dense cluster of basic amino acids (62, 67, 68). Sgk does not contain a complete NLS, but there is a short stretch of basic residues between amino acid residues 29 and 32 (KQRK) that has some NLS-like qualities, and linkage of a canonical NLS at the C terminus of Sgk (forming NLS-Sgk) was necessary to force this protein to reside exclusively in the nucleus in serum-treated cells. Similar to Sgk, several of the Ser/Thr protein kinases that shuttle into the nucleus and the cytoplasm in a stimulus- or cell cycle-regulated manner is likely to be functionally linked to the shuttling process is likely to be functionally linked to the regulatory molecules acting on Sgk, the mechanism of nuclear-cytoplasmic transport, and the compartment-specific regulators and substrates of Sgk that allow this protein kinase to be involved in both proliferative and anti proliferative signal transduction pathways in normal and transformed cells.

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